The chemical preservation of hay.

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THE CHEMICAL PRESERVATION OF HAY

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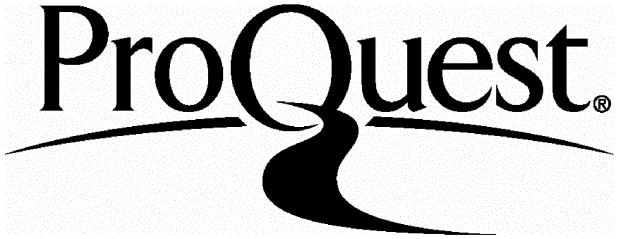

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SUMMARY

A comparison of various parameters for estimating the extent of the microbial deterioration of hay was made, these parameters being viable microbial counting by dilution plate and air sampling techniques, viable and non-viable microbial material estimates by chitin and diaminopimelic acid level determinations and finally temperature measurements. The inhomogenous nature of hay created considerable sampling errors and it was therefore concluded that heat production, expressed as degree days, was probably the most convenient measure of microbial activity.

A microbial succession was found to occur in deteriorating hay which frequently caused two separate heating phases, and it was concluded that in the first phase readily available nutrients were utilised and the second phase was due to microbial metabolism of nutrients produced by extra cellular enzymes especially cellulases.

Several chemicals and chemical mixtures were assessed as hay preservatives by three methods. Firstly, using chemically defined agar media buffered at p.H values of 5,6,7 and 8. Secondly, by their application to re-wetted hay stored in dewar flasks and thirdly by their application to hay at baling.

It was found that higher levels of these chemicals were needed on hay, than in agar media, to inhibit microbial growth and certain compounds, e.g. formaldehyde, were inactivated on hay. Also the minimum effective levels of preservative were found to increase with an increase in the moisture content of the hay and it was concluded that propionic

acid was the most practical preservative, the addition of sorbic acid possibly being of some benefit.

There appeared to be three problems concerned with the chemical preservation of hay. Firstly, poor preservative distribution, secondly preservative loss by evaporation and microbial degradation and thirdly a lack of information relating the minimum effective levels of preservative with nutrient and water content or, probably more important, the water activity of hay. Possible means of overcoming these problems are discussed.

INTRODUCTION

SECTION A - THE BIODETERIORATION OF STORED ANIMAL FEEDS

a) Introduction

The most suitable agricultural system for feeding livestock is based upon grass because, firstly it is the most economical fodder available due to its natural occurrence and its ability to grow in most soils and climates, (Grigg 1974). Secondly it provides a complete balanced diet and thirdly it can be grazed as it grows, which removes the necessity of harvesting as is the case with most fodders, for example grain.

There are three main regions in the world where grass plays an important part in the agricultural system, the ranching areas being excluded for reasons described shortly.

Firstly in Europe, the heavy soils of Cheshire and Somerset, the polders of Friesland and South Holland, the pastures of Brittany and Normandy and 80% of Norway and Finland are utilised for growing grass for feeding livestock, mainly because these areas are not suitable for other crops. In addition grass produces most of the feed in Switzerland. Secondly in North America, the dairy regions in New England and New York state graze their cattle on grass in the summer feeding them in stalls in the winter on hay, grain and concentrates. Thirdly many areas of Aust--ralasia also depend upon grazing to feed their large numbers of cattle and sheep.

Most of the world's agriculture which relies upon grass as its main livestock feed is situated in the temperate regions where during the winter months the grass stops growing and therefore a winter feed

is required. The main exception to this rule is the north island of New Zealand where the grass grows all year but this growth declines during the winter months. North Western Europe and North America does not have as long a grazing season and there are few regions where livestock can graze for more than six months of the year.

Therefore, wherever livestock are kept there is a need for large quantities of feed which can be stored throughout the winter. The solution to this problem of economically providing winter feed, is to try and preserve grass in some way throughout the winter so that it can be used to provide the livestock with a balanced diet throughout this period.

Grass which is to be used as winter fodder is grown in the summer then cut, dried and stored as hay. Hay is a complete diet for livestock as it contains not only utilisable carbohydrates in the form of sugars and cellulose and considerable plant protein (2 - 18%) but also many minerals, vitamins A, B, C and E and vitamin D develops in hay that is left in the sunlight (Watson and Nash 1960).

Hay can be used for feeding cattle, sheep, pigs, horses and green hay has been used as an essential addition of greenery to the cereals and high protein foods which formed the bulk of poultry diets. The main use of hay, however, is to feed dairy cattle throughout the winter because the demand for milk is as high in the winter as it is during the summer.

The only important agricultural system which produces livestock without the use of winter food is in the ranches of the United States,

South America and Australia where the majority of livestock reared depend almost entirely on natural pastures, and therefore this system is not included in this discussion.

Despite the obvious need of winter feed for livestock, there was little use of winter fodder until approximately 1800 when a few farmers around London fed their cattle, in stalls during the winter, on turnips, hay and brewer's grain. Hay was slowly adopted through the 16th, 17th and 18th centuries and by the end of the 18th century its production had been made easier by improvements in the scythe and the invention of the horse-drawn hay-rake. By the mid-19th century farmers had horse-drawn grass-mowing machines for hay-making and later in Western Europe the tedder became available. About the year 1880 there was a drop in world grain prices and in the United States, Western Europe and Australasia at the same time milk prices increased with the result that, local grown grain and grain imported from the Argentine and the United States were used more commonly as a winter feed. Also in Germany and Hungary by 1860 there were experiments in silage making, however, throughout this period and up to the present day, hay has remained the most important winter feed in the world, for livestock. (Grigg 1974 p 190 - 204).

Originally hay was made by cutting the grass, usually in June or July, because at this time of the year better quality hay was obtained than if the grass was cut later. It was then left lying in the field to dry and when sufficient moisture had been lost, the hay was piled up in stacks. It soon became obvious to farmers that if the hay was too damp when it was stacked it heated, became mouldy and lost some of its nutritional value and therefore methods were devised to increase the drying rate of the grass. These early methods are described by Watson

and Nash(1960) p 84 and are summarised below.

One of the earliest and simplest hay dryingaids was a device known in Europe as a 'Heinze' which consisted of vertical sharpened stakes driven into the ground with three or more horizontal bars attached. The hay was laid over the bars and built around the stake to give a heap of hay resembling a fir tree in appearance.

Another early device was a tripod or pyramid where three poles which were hinged at the top were kept apart by three horizontal bars which were fixed a foot from the ground. The hay was heaped around the tripod and this permitted good air circulation. A developement of this was the Proctor tripod, which was a metal tripod with three triangular corrugated iron vents added at the bottom of each leg which allowed currents of air into the centre of the tripod.

A device more popular on the continent than in Britain was the hurdle or 'hutte' where two hurdles each consisting of two vertical poles with two or three horizontal bars were leant against each other and the hay was built on these, angled so that rain ran off the material.

The final early hay drying aid to be discussed was known as a rack and it was particularly popular in Switzerland and Scandinavia where permanent racks were built consisting of a number of wooden or wire rails built into a permanent framework. The hay had to be carried to these racks, which was a disadvantage, and therefore portable racks became generally more popular and these were simply a number of pointed stakes driven into the ground, which were connected with wooden rails or metal wire.

A further method for drying hay, known as brown hay, was developed in England in the late nineteenth century and it then spread to Europe. Brown hay was made by placing damp hay in small stacks about 24 hours after cutting, then after it had heated and turned brown, hence the name, the hay was spread over the ground and the heat evaporated off some of the water. Finally the hay was stacked. This last method had the disadvantages of promoting the growth of micro-organisms in the hay and reducing the nutritional value of the material.

All these drying methods had the advantages of reducing the mechanical losses of the hay because it was gathered from the ground with a high moisture content, and also of reducing the leaching of nutrients from the hay by rain which can be very important, for example 50% of the starch equivalent has been removed from hay by this process.

The introduction of the pick-up baler was an important developement in hay-making and its use became widespread in the 1930's. The advantages of baling hay as opposed to stacking it, were ease of handling and storage which in turn meant smaller mechanical losses, and because air could circulate between the bales, moisture and heat were more rapidly lost from the hay when it was in store and this resulted in the hay being less prone to self-ignition. In addition, because hay was more compact in bales and in a smaller mass than in a stack, it could be stored at higher moisture levels without heating, which also reduced mechanical losses.

With present day hay-making the main problem is still the economical removal of sufficient water from the crop before it is placed in storage, in order to prevent microbial spoilage which causes the developement of health hazards to man and animals and the loss of

nutrients, both of these topics being discussed in more detail later.

Current techniques for overcoming this problem are reviewed by Zimmer (1973) and Klinner and Shepperson (1975) and are outlined below.

After grass has been cut in the field the thick stems will dry more slowly than the leaves and therefore machines which break down the surfaces of the thicker parts of the plants by crushing and crimping with pressure rollers or by laceration with flails, in order to reduce their resistance to loss of sap moisture, have been developed, to even out the drying rates between stems and leaves. It has been shown that these machines which change the physical state of the hay crop by bruising, lacerating, splitting and cutting, hasten the rate of moisture loss in the field (Klinner and Shepperson 1975).

The chopping of hay into short lengths also increases the drying rate, but this process is still in the experimental stage and has the disadvantage of rapid heating if the material is slightly too moist. Buildings have been designed for the handling, drying and feeding of chopped hay on farms, but most are no longer used because of problems of low capacity, uneven distribution of the crop within the barns, and the difficulties of matching drying rates to harvesting rates (Klinner and Shepperson 1975).

Regular turning of the grass after cutting makes little improvement in the drying rate in good conditions, but in unfavourable weather the drying rate is increased by turning up to four times per day (Westendorp 1963).

The barn drying of baled hay has increased only slowly in the United Kingdom over the past fifteen years. With the various methods used, air is passed over bales of hay, arranged in different ways, most of it flowing between the bales, however, there is usually enough air movement through them to prevent heating.

The use of chemical conditioning treatments has also been studied as a means of increasing the drying rate of hay. The effectiveness of paraquat as a pre-cutting dessicant has been disappointing, however, increased drying rates have been obtained by applying formic acid, propionic acid and mixtures of these acids to the standing crop at rates of 0.5 - 1.0% (Zimmer 1973).

Dehydration of grass is without doubt the optimal method of conservation, however, there are three main problems, namely investment, energy and organisation. An alternative to drying the grass for preservation is to ferment it wet i.e. the manufacture of silage (Watson and Nash 1960, Woolford 1972), however, this method has the disadvantages of increasing construction costs and silage does not have the nutritional value of good hay.

Finally we are left with the chemical preservation of moist hay. This technique has the advantage of allowing the hay to be handled with a higher moisture level than untreated hay, which in turn reduces the mechanical losses of the material and leads to higher nutritional values because drying, even in good hay-making conditions, can lead to a 20 to 25% dry matter loss of the fresh crop (Watson and Nash 1960 p 120). The main disadvantage of this method is that, although large quantities of high-moisture grain are stored in Europe and the U.S.A. without deterioration after the addition of chemical preservatives, usually propionic acid, results with similarly treated hay have so far been

less clear cut, and further developemental work needs to be done in this area.

b) The biodeterioration of hay

i) Microbial changes

In Britain approximately eighteen million tons of grass are conserved each year, 80% of this as hay. Of this about one third becomes mouldy during storage, mainly because it was baled too damp, i.e. with a moisture content above 20%. It can therefore be seen that the moulding of hay is a very important problem not only because of the loss in nutritional value of the hay but in addition there is the health hazard to the workers who handle the material and the animals which eat the hay.

Without doubt, a considerable amount of research needs to be done into the moulding of hay to obtain a clearer picture of what exactly happens when hay deteriorates, because although informative reports have been published in this field, still very little is really known about this problem.

Gregory et al (1963 b) studied the moulding of hay in considerable detail, baling hays at different initial moisture levels and following the temperature, microbial and biochemical changes. They concluded that the initial moisture level was the most important factor determining the changes which took place in the hay after baling.

They found that good hay which had been baled with a moisture content of 16% or less, heated little, and contained a small but diverse microflora, usually less than 10^6 spores /g of dry hay.

Hay baled with a moisture level of 25% heated to 46°C but the

fungal spore counts remained low consisting of Aspergillus glaucus spp, Cladosporium spp and only a few thermophilic fungal spores were detected. Actinomycete spore and bacterial numbers changed little in these hays.

Hay baled at 30% moisture heated to 50°C and contained mesophilic and thermophilic fungal spore counts of 10^5/g of dry hay, mesophilic and thermophilic actinomycete spore counts of 10^4/g of dry hay, mesophilic bacterial numbers reaching 10^8/g of hay and thermophilic bacterial numbers of 10^7/g of dry hay.

Hay baled at 39% moisture heated to 59°C. The fungal spore numbers reached 27 x 10^6/g of hay and consisted mainly of thermophilic and thermotolerant fungal species including Aspergillus spp, Absidia spp Mucor pusillus, Thermomyces lanuginosa, T. stellata and later during storage Paecilomyces variotii. The actinomycete spore levels reached 1.3 x 10^8/g of dry hay and included spores of Streptomyces griseus, S. griseo-flavus, S. Olivaceous, S. thermoviolaceous, Thermoactinomyces glaucus, T. vulgaris and Micropolyspora faeni.

Hay baled at 46% moisture heated to 60°C. The spore numbers increased rapidly to 10^8 fungal spores /g of dry hay and to between 10^8 and 10^9 actinomycete spores /g of dry hay. The fungal species present were similar to those in the 39% moisture hay and the actinomycetes were dominated by the thermophiles Thermoactinomyces vulgaris and Micropolyspora faeni.

Gregory et al (1963b) did little bacterial identification, but they noted that with drier hays containing approximately 20% moisture, the general aerobic count reached 2.33 x 10^6 /g of dry hay. There were several bacterial species present most of which grew at 25°C but

not at 40°C or 60°C.

In wetter hays containing 30% moisture the bacterial numbers incubated at 25°C did not fluctuate despite considerable temperature changes in the hay from which they were isolated. This bacterial population was very mixed. Bacteria capable of growing at 40°C were scarce at baling but their numbers rapidly increased as the hay heated and those capable of growing at 60°C did not appear in large numbers until the hay had heated to 59°C, the dominant bacterium at these higher temperatures being identified as Bacillus licheniformis which formed flat, thin, white overlapping colonies with filamentous margins, when incubated in nutrient agar.

Lactobacilli and anaerobic bacteria were detected in most heated hays but usually in comparatively low numbers.

The deterioration of the hays described above are only examples designed to show the effect of baling moisture level on the biodeterioration pattern of hay. Different hays baled at the same moisture level did not necessarily heat to the same degree or produce the same microbial spore population because other factors, including the type of grass, storage conditions, time of year of baling also determined the biodeterioration of the hay.

The microbiology of stacked hay was also studied and the stacks were found to form a central core of acid brown hay which contained few fungal and actinomycete spores but high numbers of bacteria reaching 10^8/g of hay and these bacteria grew at 25°C, 40°C and 60°C. The anaerobic bacteria increased to 10^7/g of hay which was higher than

usually occurred in baled hay suggesting anaerobic conditions were formed in the centre of the stack probably due to the greater mass of hay in a stack, than in a bale. The hay towards the outside of the stack contained a similar microflora to baled hay.

Gregory et al (1963b) observed microbial successions which occurred in deteriorating hay and an example was quoted, where hay containing 46% moisture was studied, because its moisture was particularly uniform.

After baling, the temperature immediately started to increase and reached 48°C after 24 hours, this phase probably being mainly due to plant respiration. The temperature then fell to 40°C after 48 hours and during this time Mucor pusillus had been developing. The temperature then rose to 57°C after 72 hours where it stayed until day six, spores of Absidia ramosa appearing during this period. As the temperature started to decline after the sixth day, spores of the thermophilic actinomycetes Thermoactinomyces vulgaris and Micropolyspora faeni appeared and they reached a maximum after eleven days. At the same time, spores of the fungi Thermomyces lanuginosa and Aspergillus fumigatus appeared. By day 14, the hay had cooled to 50°C and the fungi, A. glaucus spp, A. nidulans, Penicillium spp and Paecilomyces variotti appeared along with many mesophilic actinomycetes. Large numbers of mites also were observed and it was thought that their foraging could have accounted for the lower fungal spore counts obtained from later samples.

It is interesting to note that the phycomycetes Mucor pusillus and Absidia ramosa were the first storage fungi to appear, possibly because they were able to grow and sporulate in heating hay more rapidly than the other fungal species present. However, their spore

numbers soon declined which may have been because the available sugar resources were exhausted and cellulose became the main carbon source. This phenomenon of fungal succession in deteriorating vegetation is discussed in more detail later in this report.

ii Biochemical changes

The biochemical changes which take place when hay heats to self-ignition have been studied by various workers (Currie and Festenstein 1971, Festenstein 1966 and 1971 and Rothbaum 1963).

During the wilting of the grass the non-reducing sugars including sucrose and fructosan slowly decreased whereas the reducing sugars, including glucose and fructose increased, this trend continuing for the first few days after baling. After this stage the sugars galactose, arabinose and xylose appeared due to polymers, including hemicellulose being broken down, hemicellulose being degraded before cellulose. During this second stage proteolytic activity caused an increase in soluble and volatile nitrogen compounds which in turn raised the p.H of the hay to between 7.0 and 8.0, and mouldy hay often had a distinct odour of ammonia. When the non-reducing sugars reached very low levels the reducing sugar levels started to decrease, and by statistically correlating microbial numbers to sugar levels, Gregory et al (1963b) concluded that non-reducing sugars were more readily attacked by micro-organisms than reducing sugars.

To determine the cause of these changes, Festenstein (1966) sterilised hay with propylene oxide and followed the biochemical changes. He found the soluble nitrogen compounds increased, probably due to the

action of plant proteolytic enzymes. Pentosan, cellulose, fructosan and glucosan were all slowly broken down to their respective sugars and sucrose molecules were cleaved to glucose molecules showing the presence of invertase. These results suggest that many of the initial biochemical changes which occur in hay are due to plant enzymes and that micro-organisms are responsible for changes that occur after the first few days of storage.

It was generally agreed that microbial activity was responsible for the heating of hay up to approximately 70°C and that above this temperature exothermic chemical reactions produced the heating. Currie and Festenstein (1971) demonstrated the special conditions that were necessary for the heating of hay to temperatures above 70°C. Dewar flasks were packed with hay containing 42 - 45% moisture, and air was passed through the hay, with a relative humidity of 95 - 97% in order to prevent cooling due to water evaparation. The dewar flasks were placed in ovens and by connecting thermocouples inserted in the hay to the oven heater switches, they ensured the air surrounding the flasks was only a degree or two below the hay temperature.

Oxygen uptake and carbon dioxide release were found to be rapid as the hay heated from 40°C to 60°C, but after that phase they slowed down, so that the aeration rate could also be lowered. As the hay reached 65°C drier air was passed through the hay to remove excess water because too much moisture would have created anaerobic conditions and greater heat conductivity within the hay. Both these factors would have reduced heating.

The chemical mechanisms of the heating of hay above 70°C were

studied in more detail by these workers and it was concluded that three main types were responsible.

Firstly, if dry glucose was heated to 90°C and the heat removed, it cooled down again, but damp glucose treated similarly kept on heating with the rapid evolution of carbon dioxide. (Festenstein 1971, Rothbaum 1963). This reaction could apply to many of the free sugars present in hay. It was also noted the soluble carbohydrates disappeared rapidly when hay heated above 65°C (Currie and Festenstein 1971).

Secondly, it was thought heat could have been produced by reactions between free radicals and water vapour (Festenstein 1971).

Finally, the thermal cleavage of larger molecules including sucrose, pentosan, hemi-cellulose and cellulose. (Festenstein 1971).

If the heating of hay in a stack is compared to the heating of hay in dewar flasks, there are a few important conditions which occur in the haystack, but not in dewar flasks, which have to be taken into consideration.

In a haystack, the inside heats but the outside temperature remains constant, therefore water vapour passes outwards and condenses on the outside thus raising the temperature and creating a heat insulating layer (Currie and Festenstein 1971). A less important factor is that compounds with no osmotic potential, for example fructosan and cellulose, are broken down to give sugars which raise the osmotic pressure of the water in the hay, which in turn lowers the saturated vapour pressure causing water to condense and hence heat production.

One of the advantages of storing hay in bales rather than in stacks is that bales are less prone to self-ignition, because air can flow between the bales which removes both heat and water.

c) The biodeterioration of grain and straw

The biodeterioration of corn and straw is briefly discussed because although there are differences, mainly due to the varying chemical composition of these materials, in the microbial and biochemical changes that take place when grain and straw undergo microbial spoilage, when they are compared with hay spoilage, the three systems have many similarities. It seems probable that information about grain and straw deterioration would help in the understanding of the processes involved in hay deterioration.

i) Biodeterioration of grain

A considerable amount of work has been done on the biodeterioration of grain.

Christensen and Gordon (1948) pioneered the detailed study of the moulding of corn and wheat in storage. They found the dominant storage micro-organisms varied with the initial moisture content of the grain, which was stored in vacuum flasks. Their results are summarised in table one.

Initial moisture	max - temperature	Fungi present
16%	25°C	Aspergillus glaucus -sparse conidia.
18%	26°C	A.glaucus - perithecia abundant.
20%	29°C	A.glaucus- some A.candidus.
22%	50°C	Mucor spp, A.flavus, A. candidus.
24%	50°C	A. terreus, A.flavus.
26%	53°C	A. Fumigatus

Table one - Dominant storage flora of stored corn
(From Christensen and Gordon 1948)

When they examined corn taken from grain terminals, they found other common storage fungi including Fusarium moniliforme Aspergillus niger and Penicillium spp in addition to those in table one. The yeast Monila candida was very common especially in corn with high fungal spore counts, and was usually the dominant micro-organism, growing on the outside of the seeds as an inconspicuous crust.

Wheat samples were also examined and the common storage micro-organisms were recorded as being Aspergillus glaucus, A. candidus, A. flavus, Penicillium spp, Mucor spp, Alternaria spp and A-niger.

As a further experiment they inoculated autoclaved damp corn with pure cultures of these fungi and found the material heated to within 2-3°C of the maximum growth temperature of the fungi, and that the smaller the initial inoculum, the longer it took to reach that temperature.

Burmeister et al (1966a and 1966b) and Mc Mahon et al (1975).

studied the storage of ensiled high moisture corn. They showed that there was little mould growth if the moisture levels were kept below 20%. However, with corn stored at higher moisture levels there was rapid grain and microbial respiration which decreased the oxygen levels to between 0.5% and 1.0% within a few hours, the warmer the storage conditions the lower the final oxygen concentrations.

Their microbial studies recorded that yeasts appeared to be very important in stored corn as they were the main micro-organisms responsible for the utilisation of oxygen, which in turn inhibited further mould growth. Mc Mahon et al (1975) showed yeasts grew better than moulds when the oxygen levels fell below 8%, and that they grew well in an atmosphere containing only 0.5% oxygen. They demonstrated that Candida parapsilosis and Torüleopsis candida were the dominant field yeasts, whereas Hansenula anomola Candida pelliculosa and C guilliéremondiiwere the dominant storage yeasts, the latter group's numbersrising from 10^5/g of grain to 10^6 - 10^8/g of grain.

Mc Mahon et al (1975) also divided the fungal flora into field and storage types. Fusarium spp, Penicillium spp, Mucor spp, Cladosporium spp and Alternaria spp were placed in the former group, whereas Aspergillus spp and Penicillium spp were dominant in the latter group.They concluded that moulds were the primary factor in deteriorating high moisture corn, even with low oxygen and high carbon dioxide tensions, because only the mould counts increased significantly when the corn deteriorated, their total spore count rising from 10^5/g to 10^7 - 10^8/g of corn, during storage.

More recent research (Tobak and Cooke 1968) has shown that many

fungi can grow and sporulate in low oxygen tensions and this topic will be discussed in more detail later in this report.

Bacteria were also studied in relation to high moisture corn storage and it was found that their numbers increased considerably even if the material did not deteriorate. The general aerobic counts rose from 10^6/g of grain to between 10^8 and 10^9/g of grain, coliform bacteria increased from between 10^4 and 10^5 /g of grain up to between 10^6 and 10^8 /g of grain in the upper corn layers but decreased to 10 /g of grain in the lower layers, while Lactobacilli increased from 10^6 /g of grain to between 10^8 and 10^9 /g of grain (Mc Mahon et al 1975).

ii) Biodeterioration of straw

Chang and Hudson (1967a) and Chang (1967b) studied the biodeterioration of wheat straw compost.

The material they stacked, heated to 67°C - 72°C after five days storage, then the temperature fell to 40°C after a further two days when it again rose to 50°C.

They studied the micro-organisms present and found the mesophilic bacteria increased from between 10^6 and 10^7 /g of straw to 10^8 /g after four days, they then declined to between 10^6 and 10^7 /g of straw and finally rose again to 10^9 /g after twenty six days. The thermophilic bacteria increased from between 10^4 and 10^5 /g of straw to 10^9 /g after six days.

Mesophilic actinomycete spore numbers started at between 10^6 and 10^7 /g of straw, were not detected after five days and then rose to 10^6 /g after twelve days. Thermophilic actinomycete spores were initially 10^4 to 10^5 /g, they then rose to 10^8 /g after five days and finally slowly declined to 10^6 /g after forty days.

Mesophilic fungal spore numbers started at 10^6 to 10^7 /g of straw, were not detected after eight days then reappeared after twenty days, which could have been because the high temperature of the straw was inactivating them. The thermophilic fungal spore numbers were initially 10^4 to 10^7 /g but were not found after five days, however, they re-appeared to reach counts of between 10^6 and 10^7 /g after sixteen days.

Chang correlated these microbial counts with the heating of the straw and summarised his work by dividing the process into three phases.

An initial phase of mesophilic micro-organisms being active and raising the temperature to $40^{\circ}C$.

A second phase where the temperature rose over $40^{\circ}C$, the mesophiles were then [supressed] and the thermophiles became active, raising the temperature to $70^{\circ}C$, the thermophilic fungi becoming inactive at temperatures exceeding $60^{\circ}C$.

A final phase where the nutrients were exhausted, therefore microbial activity decreased and the straw cooled down.

The fungi were studied in more detail and were divided into three groups, depending on their order of appearance during storage.

GROUP ONE - (a) The primary saprophytes or field flora, including Cladosporium herbarum, Alternaria tenuis , and Aureobasidium pullulans.

(b) Initial storage fungi on material which was dry and therefore did not heat rapidly; including Aspergillus repens, A. amstelodami, A. versicolor, A. candidus, A. nidulans and Penicillium spp.

(c) Thermophilic and thermotolerant fungi on rapidly heating material including Mucor pusillus, Absidia ramosa, Aspergillus fumigatus and Thermomyces lanuginosa.

GROUP TWO - Thermophilic fungi which appeared during the 'plateau' phase of heating including Humicola insolens, Thermomyces lanuginosa, Chaetomium thermophile, Malbranchea pulchella var. sulfurea and Talaromyces dupontii.

GROUP THREE - Fungi which established themselves when the temperature fell, including the thermophiles Sporotrichum thermophile and a mycelia sterilia which was readily recognisable by its small brown sclerotia, and the mesophiles Fusarium culmorum, Stysanus stermonitis, Caprinus cinereus, C. megacephalus and Clitophilus pinsitus.

(d) A Microbiological comparison of deteriorating hay, grain and straw

A comparison of the microbiological studies of deteriorating hay, grain and straw shows several similarities. The storage fungi are dominated by mesophilic Aspergillus and Penicillium species unless the material has heated sufficiently in which case thermophilic and thermotolerant fungi become dominant including Mucor pusillus, Absidia

ramosa, Aspergillus fumigatus, Thermomyces lanuginosa, Chaetomium thermophile and Malbranchea pulchella var sulfurea. Mesophilic bacteria and actinomycetes will only increase if the material does not heat to high temperatures in which case the thermophilic bacteria and actinomycetes become numerous.

The fungal succession that occurred in straw was similar to that which occurred in hay (Gregory et al 1963b), and a suggested explanation for these successions is that the initial storage micro-organisms grow rapidly utilising the readily available soluble nutrients. Their activity combined with respiratory activity of the plant material causes heating which allows the phycomycetes Mucor pusillus and Absidia ramosa to grow and sporulate rapidly. Eventually these nutrients become exhausted and larger molecules including cellulose and hemicellulose are degraded by the cellulolytic fungi including Aspergillus fumigatus, Chaetomium thermophile and to a lesser extent Malbranchea pulchella var. sulfurea. The non-cellulolytic Thermomyces lanuginosa and Talaromyces dupontii are secondary saprophytes which utilise the sugars produced by the cellulolytic fungi. The cellulolytic studies in this work are supported by the reports of Tansey (1971) and Fergus (1969). Why Mucor pusillus and Absidia ramosa do not grow as secondary saprophytes is unclear but it maybe because, as both straw and hay deteriorate, their p.H values increase which would tend to inhibit the growth of the phycomycetes which prefer more acidic conditions than the other thermophilic fungi (Rosenberg 1975).

It is interesting to note here, the work of Hedger and Hudson (1974) where T. lanuginosa was grown with other thermophilic fungi on

cellophane and filter paper. They found that with Humicola insolens, Chaetomium thermophile, A. Fumigatus and Malbranchea pulchella var sulfurea, the T. lanuginosa grew sparsely until it met the above fungi and then it grew rapidly and sporulated.

The utilisation of various carbon sources may not have been the complete answer to these fungal successions, but because the major part of the weight loss of straw was due to hemicellulose and cellulose degradation (Chang 1967b), it appears likely to have been an important factor, along with others including oxygen depletion, carbon dioxide accumulation and temperature variations.

e) The importance of water in biodeterioration

The important role that water plays in hay, straw and grain deterioration has been discussed earlier. It is, however, not only the absolute amount of water present which is important, but the availability of this water.

Fresh green grass which has been cut, dried and baled, may, for example, contain 30% moisture. If this grass is compared with old hay or grass cut late in the summer which also contain 30% moisture, the former will probably have less available water because there are more soluble compounds, especially sugars present (Watson and Nash 1960) which would reduce the chemical potential of the water. It is the same principal which preserves commodities including jams, which contain high sugar levels. In practice fresh hay tends to heat more than old hay probably because the extra nutrients available stimulate microbial growth by more than the lower available water inhibits microbial growth. However, when considering the deterioration potential of hay, the

amount of available water should be considered rather than the absolute amount.

The term most commonly used to measure this available water, is water activity (Aw) which is defined as, the ratio of the vapour pressure of the water in a solution or a hygroscopic material to that of pure water at the same temperature and pressure.

In a review on this subject, Ayerst - (1965) discusses water activity in more detail and mentions methods of measurement.

Nearly all the work on hay deterioration in the literature is concerned with actual moisture content expressed as a percentage of the total weight of hay, whereas, when considering microbial growth it is the water activity which is important, and this will vary at a given moisture content between different types of hay.

The works of Scott (1957) and Ingram (1957) show that, in general, fungi can grow at lower water activities than bacteria. Ayerst (1969) studied the relationship between temperature and water activity concerning the growth storage fungi, and concluded that the fungi would grow at the lowest water activities at temperatures close to their optima. He noted that some fungi, usually Aspergillus glaucus group, would not grow at the highest water activities studied but grew well at lower values. This phenomenon was used to explain why these fungi sometimes grew on stored grain at higher temperatures than those at which they would grow on normal agar media, the agar having a high water activity compared with grain.

Ayerst (1969) grew Aspergillus chevalieri and A. amstelodami

at water activities as low as 0.71, although A. nidulans grew at a water activity of 0.78 this being the lowest value for a common hay fungus. A. glaucus spp and A. nidulans can grow at low water activities and this probably partly determines why they are common in drier hays, whereas bacteria are considered to be important, only in the deterioration of wetter hays, because they require higher water activities for growth.

f) Diseases caused by mouldy hay

i) Human diseases

Many workers have concentrated their research on the clinical problems associated with mouldy hay. It is a well known fact that humans handling mouldy hay can breath in large numbers of microbial spores because in a barn where mouldy hay was being handled, spore counts have shown that their numbers can reach 2.9×10^9/ metre ^{3} of air (Lacey et al 1972). These spores when inhaled can cause respiratory complaints, the most familiar and well studied being the allergenic disease known as Farmer's lung.

Farmer's lung is described as a respiratory complaint in humans who have become hypersensitive to the antigens in dust from mouldy hay. The hypersensitivity increases with repeated exposure, and in many cases limited exposure may provoke severe symptoms. The clinical symptoms of Farmer's lung are that it commences with chills, general malaise, fever and shortness of breath which occurs a few to several hours after exposure to mouldy hay. These symptoms usually last for two to three days but can occasionally last longer.

Gregory and Lacey (1963a) studied the spores released by good hays

and hays known to have caused Farmer's lung. The latter hays it was concluded, contained many more spores of the thermophilic fungi Aspergillus fumigatus and Thermomyces lanuginosa and of the thermophilic actinomycetes able to grow at 60°C. They concluded that mouldy hay contained spores which were potentially hazardous to man including those of the fungi Absidia spp, A. fumigatus Mucor pusillus, Thermomyces stellata and Scopulariopsis brevicaulis and those of the thermophilic actinomycetes Thermoactinomyces vulgaris and Micropolyspora faeni.

Before these spores can cause illness, including Farmer's lung, they have to be able to penetrate the lungs and preferably deep into the alveoli. Druett (1967) noted that the larger the particles the less likely they are to penetrate the lungs and therefore the more that would be needed to cause infection. Lacey et al (1972) considered spore size and its relationship with lung penetration and concluded that particles larger than 10 μm in diameter would be trapped in the nose and may cause rhinitis, particles 4 μm to 10 μm in diameter would be deposited in the bronchi and bronchioles and cause asthma while spores with diameters less than 4 μm would penetrate the alveoli and may cause alveolitis. Lacey et al (1972) also recorded that most of the fungi which grow in hay, produce spores with diameters between 2 and 4 μm and that the thermophilic actinomycete spores have diameters less than 2 μm. It would appear, therefore, that the majority of spores released from deteriorated hay, would be capable of penetrating deep into the lung alveoli, especially in the case of the thermophilic actinomycetes.

Lacey (1975) considered the relationship between different micro-organisms and the various diseases they could cause, in further detail,

and divided the fungi and actinomycetes which cause infection into two groups.

Group A - those causing immediate allergenic reactions, including Cladosporium herbarum, Alternaria tenuis, Ustilago spp and Aspergillus fumigatus.

Group B - those causing delayed (for several hours) allergenic reactions including A. fumigatus, A. clavatus, Penicillium spp Micropolyspora faeni and Thermoactinomyces vulgaris.

The baling moisture content of hay was correlated with the potentially hazardous micro-organisms which were likely to develop (Lacey 1975).

Hay baled at 15 - 25% moisture contained Aspergillus repens and A. amstelodami, both being allergen and toxin producers.

Hay baled at 30% moisture contained A. versicolor which has been implicated in 'Farmer's lung' and the production of the toxin sterigmatocystin.

Hay baled at 35% moisture contained A. nidulans, Absidia spp and Mucor pusillus all of which are pathogenic.

Hay baled at 40% moisture contained the thermophilic actinomycetes which cause 'Farmer's lung' and A. fumigatus which is responsible for aspergillosis.

Pepys et al (1963) carried out serological studies in order to

determine which micro-organisms present in hay were responsible for causing 'Farmer's lung'.

They inoculated sterile hay samples with various micro-organisms responsible for the moulding of hay. After incubation they made extracts from these hays which were then tested against sera from patients suffering from Farmer's lung.

Extracts from hay on which Mucor pusillus, Absidia ramosa, Aspergillus fumigatus, Thermomyces lanuginosa, T. stellata and Paecilomyces spp had grown, reacted with the sera, but this reaction was found to be due to fungal antigens and not 'Farmer's lung' antigens. The only extracts which provided 'Farmer's lung' antigens were those from hay which had been treated in one of three ways.

1) Using mixed microbial suspensions from antigenically active hay.

2) Using mixed suspensions of pure cultures of thermophilic actinomycetes from hay, where the p.H of the hay had been raised to 7.0 either by prior inoculation with fungi or infiltration by ammonia vapour.

3) Inoculation with pure cultures of Micropolyspora faeni or to a lesser degree Thermoactinomyces vulgaris, again using hay with its p.H raised to 7.0.

It was concluded that Micropolyspora faeni was the richest source of Farmer's lung antigen and that the growth of micro-organisms was necessary on hay, to raise the p.H value, before the thermophilic actinomycetes were able to grow.

'Farmer's lung' is not the only disease that can be caused by spores from mouldy hay, there are many others some of which have briefly been discussed earlier. Probably the most serious is aspergillosis

in humans where spores of Aspergillus fumigatus can lodge in the lungs and germinate to cause aspergilloma. (Lacey et al 1972, El - Am 1975). This infection is very difficult to cure and can cause serious illness.

Similar respiratory complaints to Farmer's lung are caused in humans who have been handling various mouldy materials, these include grain fever from mouldy grain (Aspergillus spp and Penicillium spp) Bagassosis from sugar cane bagasse (Thermoactinomyces sacchari), Sequoiosis from redwood sawdust (Aureobasidium pullulans and Graphium spp), Maltworker's lung from malting barley (Aspergillus fumigatus and A. clavatus) and cheese Worker's lung from cheese (Penicillium casei), the causative micro-organisms being bracketed, (Lacey et al 1972).

ii) Animal diseases

Animals which eat mouldy hay can contract diseases, aswell as the humans who handle it.

Cattle which eat mouldy hay may catch fog fever (Lacey/1968) and precipitans of Aspergillus fumigatus were found in the sera of animals suffering from this disease. Austwick (1963) showed that the lungs of cattle suffering from sub-clinical aspergillosis contained lesions which had asteroid bodies enclosing viable hyphae of A. fumigatus. He also injected 3×10^7 spores of this mould into pregnant heifers and they contracted a uterine infection, however, when similar animals inhaled 3×10^9 spores then no infection occurred. Certain fungal spores appear to be potentially pathogenic, however, very large numbers are needed to cause infection in healthy men or animals and 10^8 spores per day can be inhaled by a man, without any apparent harmful effects.

(Austwick 1963).

Mycotic abortions are often attributed to micro-organisms found in mouldy hay. Sulochana (1970) experimentally infected pregnant cows with A. fumigatus and Absidia ramosa and caused many of them to abort. In experiments where cattle had had abortions but none of the usual abortion agents were present, including Brucella, Vibrio, Trichomonas and Leptospira, the fungi A. fumigatus, A. terreus, A. flavus, Scopulariopsis and Blastomyces were isolated from the abortions. Austwick (1963) calculated that 13 - 25% of cattle abortions could be attributed to these fungi.

Pulmonary emphysema (broken wind or heaves) in horses is another condition attributed to micro-organisms from mouldy hay, and is very rarely found in animals which have been kept in pasture. Eyre (1972) showed that infected horses, which make an exaggerated double respiratory sound, gave marked skin reactions following intradermal injections with mixed fungi. Nine out of ten horses tested with single fungal antigens showed positive reactions to A. fumigatus and also to Alternaria spp. Three horses were tested with Penicillium antigens and all showed positive though weak reactions. Ten symptomless horses were also tested, only one reacted to A. fumigatus, three gave weak reactions to Alternaria and another showed moderate Penicillium hypersensitivity. Eyre (1972) also removed ten affected horses into air conditioned isolation in boxes bedded with wood shavings and fed them on a hay and straw free diet. In all cases after 48 hours the symptons had either disappeared or markedly decreased. When these horses were returned to the dusty stable area symptoms of "Broken-wind", returned, thus leading to the conclusion the disease was caused by an

air-borne allergen.

Aspergillosis caused by A. fumigatus is a serious disease in young chicks where post-mortem examinations of infected animals have revealed heavy growth of the fungus in the lungs. Austwick (1963) stated that 10% of the deaths of chicks, under 14 days old, were caused by this fungus.

iii) Toxins produced

The disease aspect of hay is not only confined to the direct action of the micro-organisms on the bodies of man and animals, but also to the toxins which are produced by many of the fungi. These fungal toxins are collectively known as mycotoxins, the best known being called aflatoxin, which is produced by Aspergillus flavus. Watson (1976) outlines the current problems of mycotoxins in feed.

The literature on mycotoxins especially aflatoxin is copious to say the least, but Lacey (1975) has produced a fine review article, in which he lists the best known mycotoxins and the fungi which produce them. The main points he makes are listed below.

Aflatoxin produced by A. flavus and Penicillium islandicum.

Ochratoxin produced by A. ochraceous and P. viridicatum, this toxin acts on the liver and kidneys.

Stachybotrys atra and Fusarium tricinctum cause haemorrhagic disease.

Dendodochium toxicum affects the nervous and cardiovascular systems.

Penicillium cyclopium attacks the central nervous system and causes tremors.

Gibberella zeae produces zearalenone which acts oestrogenically and causes infertility.

Fusarium spp produce metabolites which cause vomiting and diarrhoea.

Some fungal species may even work together, for example Fusarium spp grow on ergot sclerotia and convert lysergic acid containing alkaloids like ergotamine to lysergic diethylamine (L.S.D).

In a review article by Cane et al (1968) they recorded that aflatoxins are harmful to a whole range of animals including in order of sesitivity, trout, ducklings, turkeys, young pigs, pregnant sows, cattle and sheep. Typical symptoms of aflatoxin poisoning are a yellow colouring in the eyes and orange-yellow urine in livestock. A post-mortem may show nodules and a yellow colour in the liver, and that other organs and even the tissue could be yellow. Haemorrhages may also be found in the stomach and other parts of the body.

Cane et al (1968) list the conditions necessary for the production of aflatoxin in animal feeds.

(1) A strain of A. flavus capable of producing the toxin.

(2) A moisture content of 18.5% or more, this being in equilibrium with a relative humidity of 85%.

(3) A temperature range within 12°C to 40°C, the optimum being approximately 27°C.

(4) Time, with optimum conditions for growth of the fungus some aflatoxin may be produced within 24 hours and maximum production is reached within 4 to 10 days.

Fortunately A. flavus is not usually found in mouldy hay,

although the other three conditions are adequately fulfilled by most hay baled in this country.

To demonstrate the damage that aflatoxin can do, the case where 100,000 turkey poults died in England in 1960 should be examined. Research demonstrated that the so called "Turkey -X- disease" was caused by an aflatoxin produced by an A. flavus strain which had contaminated a peanut meal shipment. The financial loss was estimated at £133,000.

The mycotoxins are a more serious problem in animal feedstuffs from hot humid climates, including peanut meal, cottonseed meal, copra and fishmeal from Africa, South America, and Southeast Asia and even grain stored in Great Britain usually contains more mycotoxins than hay. The danger of poisoning from mouldy hay should not, however, be ignored, especially after the work of Davis et al (1975). This shows that many of the thermophilic fungi found in mouldy hay produce unidentified toxins which were fatal to Brine shrimps and chick embryos and caused a loss of weight in rats.

The fungi Thermomyces lanuginosa, Mucor pusillus, Aspergillus fumigatus, Chaetomium thermophile var caprophile, Talaromyces thermophilus, Thermoascus auranticus and Acremonium alabamensis produced toxins in chemical media, which killed a significant proportion of chick embryos. All these fungi, except Talaromyces thermophilus and Thermoascus auranticus, produced toxin which killed at least 20% of treated brine shrimps, and Mucor pusillus was the only fungus which did not produce a toxin which reduced the body weight of rats. Extracts from Thermomyces lanuginosa even killed some of the rats tested.

From this work it appears possible that some thermophilic fungi are mycotoxin producers and a few identified mycotoxins have been found in mouldy hay (Mirocha et al 1971). These toxins may have accounted for some of the clinical symptoms which have been observed in animals which have consumed mouldy fodders including hay.

The main problem with mycotoxins is the poisoning of the animals which consume the feed, however, the implications of these compounds as being damaging to human health are probably at present being underestimated (Colin and Stoloff 1974), and care is needed, because many mycotoxins are undoubtedly extremely toxic towards man, aswell as animals.

As a final note on mycotoxins, research by Smith et al (1976) has shown that aflatoxicosis in farm animals occurs with greater incidence and severity than was previously suspected and that other mycotoxicoses may cause equal or greater problems. Concern has been shown by Watson (1976) over the advent of the large bales for hay and straw, which is likely to increase the degree of moulding in these animal feeds. There seems little doubt that the control of animal feed deterioration and hence the mycotoxin production, will be one of the important developements in agriculture.

SECTION B - THE CHEMICAL PRESERVATION OF HAY

a) The need for a preservative and its properties

Ideally, hay should be baled with a moisture content below 16% to prevent it from moulding during storage, however, if the weather is not suitable then this can not be done using conventional hay-making methods and artificial drying has proved too expensive. All this leads to the conclusion that an effective hay preservative would be the ideal compromise.

There are other advantages to baling damp hay with a preservative, namely, that nutritional losses incurred during drying and mechanical losses due to post cutting treatments would both be reduced.

There is therefore, ample evidence for the need of a hay preservative, however, before a chemical can be considered suitable, there are several important properties it has to fulfill.

1) It must be an effective antimicrobial agent, being able to inhibit the growth of the many species of actinomycetes, bacteria and fungi which are encountered in hay. A mixture of two or more compounds acting synergistically could be the most effective treatment.

2) Hay is often stored for several months and therefore any preservative must be persistent on the hay and be resistant to microbial degradation.

3) It must be harmless, after storage, to the rumen flora, This condition rules out most anti-microbial compounds, for example many antibiotics.

4) It must be easy and not too unpleasant for workers to handle.

5) It must be non-toxic to mammals, before and after storage, not only for the safety of the animals eating the hay, but also for the workers who handle the preservative, a non-toxic compound being one which has no ill effects. Extra care must be taken with dairy cattle to ensure that their milk is unaffected.

6) It must be cheap enough to make hay preservation economically viable and be available in large enough quantities to treat the nations hay crop.

7) It must be in a form whereby it can be applied to hay, either as a liquid or as a solid which can be readily dissolved in some suitable solvent, preferably water. The preservative could then be sprayed onto the hay as it entered the pick-up baler.

These conditions trim down the long list of known anti-microbial compounds, to a few potential hay preservatives.

Various compounds, which were considered during this work as potential hay preservatives, will be discussed, noting their advantages and disadvantages and possible reasons why some may work as hay preservatives more effectively than others.

b) Chemicals considered as potential hay preservatives

i) The volatile fatty acids (V.F.A.).

The V.F.A. are a group of compounds, which up to the present time, have proved more satisfactory as hay and grain preservatives than any other. V.F.A. have been used commercially as hay and grain preservatives mainly in the United Kingdom, Europe and the United States for the last few years and have proved to be reasonably effective, however, as with most preservatives, there is room for improvement.

The reasons why V.F.A. have been more effective hay preservatives than other compounds, so far tested, are outlined below with respect to the necessary properties governing such a preservative, which have been outlined earlier.

Firstly, V.F.A. are effective antimicrobial agents, being microbistatic at low levels and microbicidal at higher levels. They are effective against most micro-organisms (Nieman 1954, Hoffman et al 1939), they persist for a reasonable length of time on hay and do not react with chemical components of the hay as in the case of compounds including formaldehyde.

Secondly, they are harmless to the rumen flora and to man and mammals. This is known because V.F.A. are the main metabolic products produced in the rumen, and they then pass through the rumen wall and enter the animals bloodstream where they are transported to various organs of the body to be oxidised.

Thirdly, although V.F.A. can burn the skin and eyes, and their vapours are irritating if inhaled, they are safe to handle providing suitable precautions are taken. Unfortunately they will attack the paintwork and metal of machinery and ideally, some type of protective covering should be applied to all hay-making machines when a V.F.A. containing preservative is being used.

Fourthly, V.F.A. are prepared commercially in petroleum manufacture and are available in large quantities at reasonable prices.

Finally, V.F.A. are liquids at normal temperatures and can therefore

easily be applied as a spray to hay.

Thornton (1963) studied the anti-fungal properties of the straight chain fatty acids as shown in table two, against the fungus Pithomyces chartarum (4 strains), which causes the liver disfunction of sheep known as facial eazema and the fungi Fusarium oxysporum, F. culmorum, Cylindrocarpum radicicola, Mortierella alpina, a Phoma spp and a sterile brown mycelium.

Number of carbon atoms	Title	Volatility
C_1	Formic acid	
C_2	Acetic acid	Volatile
C_3	Propionic acid	fatty
C_4	butyric acid	acids
C_5	Valeric acid	
C_6	Caproic acid	
C_7	heptylic acid	
C_8	Caprylic acid	
C_9	Pelargonic acid	Partly
C_{10}	Capric acid	volatile
C_{11}	Hendecanoic acid	fatty
C_{12}	Lauric acid	acids

Table two - The straight chain fatty acids, (from Thornton 1963).

It was found that the vapour of the C_1 - C_7 acids completely inhibited the growth of all the fungi, that caprylic acid vapour partly inhibited the growth of all the fungi except Mortierrella alpina which was completely inhibited and pelargonic acid vapour inhibited the growth of all the fungi except one strain of P. chartarum

which was unaffected. The C_{10}, C_{11} and C_{12} acid vapours had no effect, probably because they were the least volatile.

Thornton also tested the effect of the acid vapours on spore germination of P. Chartarum and found that only formic and acetic acid vapours prevented germination, except when long exposure periods of 120 minutes were used, when propionic and butyric acid vapours also inhibited germination. These results were concluded also to be due to the greater volatility of the short chain acids.

Finally V.F.A. were included in potato- carrot extract agar, and the medium inoculated with mycelial discs of P. chartarum. The results obtained, are shown in table three.

Minimum concentrations of fatty acids as emulsions totally inhibiting growth of mycelial discs of P. Chartarum strain C. Mean of duplicate plates incubated at 27°C for seven days.

Fatty acid	Inhibitory concentration % (W/V)
C_3 C_{11} C_{12}	0.10
C_{10}	0.08
C_4	0.07
C_5 C_9	0.05
C_6 C_7 C_8	0.04

Table three-from Thornton (1963)

It was concluded that the C_6 - C_8 V.F.A. were the most active antimicrobially, but that the lower the chain length, the more

volatile the acid which caused their vapours to be more inhibitory.

Woolford (1975a) made a detailed study on the activity of the V.F.As., formic to lauric inclusive, against a range of silage micro-organisms. His results show that the V.F.A. had antimicrobial activity against bacteria, yeasts and moulds and that their activity generally increased with greater chain length and a decrease in the p.H of the environment. This work does not agree completely with the results of Fay and Farias (1975) who also demonstrated an increase in the antimicrobial activity of V.F.A. with increasing chain length but who concluded that the C_9 and C_{10} acids were the most effective and that hendecanoic acid (C_{11}) was not very effective. They were, however, using only Escherichia coli. Gram negative bacteria appear to be more resistant to the activity of V.F.A. than Gram positive bacteria (Freese et al 1973) although why this should be does not seem certain.

It is thus well established that the V.F.A. have a good general antimicrobial action against bacteria and fungi, but little has been done on their activity against the actinomycetes, which are the third important group of hay deteriorating micro-organisms. Crook et al (1953) and Durbin (1961) suggested the use of sodium propionate in chemical media to isolate actinomycetes, because their growth was not inhibited as much by this chemical, as the growth of the bacteria and fungi. It would seem possible, therefore, that V.F.A. treatment of hay could enrich for actinomycetes. Pizarro et al (1973) demonstrated that 0.1% propionic acid incorporated into chemical media completely prevented the growth of the two thermophilic actinomycetes which cause Farmer's Lung, namely Micropolyspora faeni and Thermoactinomyces vulgaris.

All the V.F.A. have potential as hay preservatives, however, the cost of valeric to lauric acids makes their use dubious. Other important points to be considered are that the shorter the chain length of the acid molecules the more volatile they become and are more likely to permeate through the hay. Also the higher acids are solids which are insoluble in water, therefore application becomes a problem and finally butyric, valeric and caproic acids have particularly unpleasant odours.

Woolford (1975a) noted that because the short chain acids dissociate to a greater extent in water, they lower the p.H of grass more than the long chain acids, an increase in the acidity of the grass being desirable for two reasons. Firstly, when grass is cut it has a p.H of 5.0 - 5.5 and if this value is lowered, the growth of bacteria, actinomycetes and some fungi would be inhibited. Secondly, many anti-microbial agents which could be used as hay preservatives are more inhibitory at low p.H values, as can be seen from the results described later in this work.

To conclude, it appears that the C_1 to C_3 V.F.A. are the most suitable for use as hay preservatives.

ii) Sorbic acid and its derivatives

Sorbic acid (2, 4 - hexadienoic acid) is a common preservative used in many foods including cheese, fruit juices, frostings, cakes, pie fillings and wine, and was considered to have potential as a hay preservative.

$CH_3 - CH = CH - CH = CH - COOH$
Sorbic acid

This chemical fulfills all the necessary requirements for a hay preservative which have been listed earlier. Unfortunately, it is insoluble in water but the application onto hay could be achieved by dissolving sorbic acid in a volatile fatty acid and spraying it on the hay. It is slowly metabolised by various micro-organisms, which is discussed later, and is therefore unlikely to harm the rumen flora.

The effectiveness of sorbic acid as an antimicrobial agent has been well demonstrated (Gooding 1945), and Smith and Rollin (1954) showed its marked superiority to sodium benzoate as a fungistatic agent in cheese and cheese products.

Finally, evidence that sorbic acid is harmless to mammals, is well documented. Bedford and Clarke (1973) fed sorbic acid to cats at a rate of 2% in their food, found no short term effects and considered it to be completely harmless. Deuel et al (1954a) went further and added sorbic acid to the feed of rats at a rate of 8% and to dog feed at a rate of 4%, In both cases no harmful effects on the animals were observed. Rats fed with material containing 8% sodium benzoate suffered adverse effects. Furthermore, sorbic acid was considered to be utilised by the mammalian body using the same metabolic pathway as for caproic acid because $\alpha\beta$ - unsaturated fatty acids are intermediates in the normal oxidation of the corresponding fatty acids (Deuel et al 1954b).

The main disadvantage of sorbic acid as a hay preservative is that it is only strongly antimicrobial at p.H values of less than 5.0.

This is thought to be because it is only active in the undissociated form, being unable to pass through the cell membrane of the micro-organisms as the dissociated molecule. In solutions at p.H 4.8, 50% of sorbic acid molecules are undissociated whereas at p.H 7.0 only 1% remain undissociated (Bell et al 1959). Although the p.H values of hay at the time of baling are usually between 5.0 and 5.5 (Gregory et al 1963b), the conditions are still not optimal for the anti-microbial activity of sorbic acid. Possible solutions to this problem would be to apply sorbic acid to the hay along with a strong acid, or to use one of the anti-microbially active derivatives of sorbic acid which remain undissociated at high p.H values.

Dudman (1963) tested such a compound, which he called sorbic hydroxamic acid, but which is now more commonly called sorbohydroxamic acid, against the fungi Aspergillus niger, Penicillium notatum a Rhizopus spp, Botrytys cinerea and Cladosporium cinerea, in grape juice. At a concentration of 0.1% this compound inhibited the growth of all the fungi for 38 days at room temperature, over a p.H range of 3.6 - 9.2, whereas, potassium sorbate, at the same concentration, only prevented fungal growth at p.H 3.6. Dudman (1963) noted that 50% of sorbohydroxamic acid molecules are undissociated at p.H 8.0.

Troller (1967) carried out a more detailed study of sorbic acid derivatives as potential food preservatives and found that sorbaldehyde and sorbohydroxamic acid were stronger antimicrobially than sorbic acid, when tested against an Aspergillus niger strain which had been isolated from food products containing sorbic acid and which was resistant to sorbic acid.

$$CH_3 - CH = CH - CH = CH - CO - NHOH$$

sorbohydroxamic acid

Troller (1967) also showed the effectiveness of sorbohydroxamic acid hardly varied between p.H values of 5.0 and 7.0, and this factor was probably the main reason why it preserved various foods more effectively than sorbic, propionic and benzoic acids, a concentration of 0.0075 M completely preventing mould growth in frostings for 216 days.

Unfortunately sorbohydroxamic acid has several drawbacks as a potential hay preservative. Firstly, it is considerably more toxic to rats than is sorbic acid, having a $L.D_{50}$ gm/kg value of 0.7 as opposed to 7.5 for sorbic acid. Secondly its metabolic fate and long term effects on the mammalian body are unknown. Thirdly, if it is persistent in the hay it could damage the rumen flora and finally it is not produced in large quantities and therefore its economic faesibility is difficult to assess, although, both sorbic acid and hydroxylamine, from which sorbohydroxamic acid is produced, are relatively cheap.

Sorbaldehyde had a strong antimicrobial activity (Troller et al 1967) but it also had an undesirable odour and flavour which ruled it out as a food preservative, but not necessarily as a hay preservative.

More work needs to be done before most of the above compounds could seriously be considered for hay preservation.

iii) Formaldehyde and Paraformaldehyde

Formaldehyde is a well known antimicrobial agent used in medicine and microbiology for sterilising many items, ranging from used microbial

cultures to whole buildings.

It has not been used entensively in animal fodder preservation, except with silage, where encouraging results have been obtained (Brown and Valentine 1972, Valentine and Brown 1973), mainly due to its unpleasant odour, its toxicity towards mammals and because it causes dermatitis. It is, however, an effective, inexpensive antimicrobial compound which could easily be sprayed on hay if dissolved in water. Furthermore, formaldehyde would be unlikely to harm the rumen flora because it would not persist, once applied to hay, in an active form for two reasons. Firstly, being volatile, it would diffuse into the atmosphere, and secondly it would combine with many compounds present in hay especially proteins. This latter quality is very important,because if too much formaldehyde was present, it would combine with a significant proportion of the grass protein, rendering it unavailable to the rumen micro-organisms and therefore indirectly to the animals themselves.

Formaldehyde was used to fumigate broiler houses between broods. (Dennis and Gaunt 1974) and its effect on the germination of conidia of several common fungi was studied. It was found that when exposed to these spores, 2 p.p.m of formaldehyde in air killed 99. 99% of them in 24 hours, however, 10 p.p.m were needed to obtain similar results for dust taken from broiler houses.

Bennet (1973) tested twelve formaldehyde releasing compounds, comparing their effectiveness at preserving cutting fluids. He concluded that 3,5 - Dimethyl - tetrahydro - 1, 3-5- thiadiazine -2-thione was the most effective, however, this compound would be too

expensive for use as hay preservative.

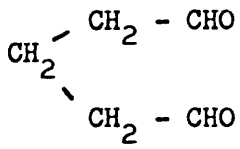
One problem likely to arise with the use of formaldehyde as a hay preservative would be its lack of persistence, due to reasons outlined above. One possible means of overcoming this problem would be to use paraformaldehyde, which is a polymer of formaldehyde which slowly decomposes in normal atmospheric conditions, giving off formaldehyde gas.

Paraformaldehyde has yielded favourable results when compared with formic acid as a preservative of the nutritional qualities of silage (Waldo et al 1975), and Woolford (1975b) also working on silage, tested formaldehyde and paraformaldehyde against a whole range of silage micro-organisms. He found the compounds were strongly anti-microbial against the whole range, including the moulds and yeasts when tested in chemical media. These latter results do not however, take into account the chemical combining of formaldehyde to chemical components in the grass.

Veloso et al (1974) treated poultry litter with 1% and 3% paraformaldehyde. They found, with the 3% treatment, the bacterial counts were reduced to 10% of the untreated value and mould counts to 1%, for up to three weeks, after which time they returned to the values of the untreated litter, the 1% treatment not being as effective. Although, formaldehyde is toxic to animals, day old chicks appeared unaffected when they lived on the treated litter, probably because the paraformaldehyde flakes slowly decomposed giving off formaldehyde which then reacted with organic matter present, to render it harmless. This work suggests the effective preservative life of paraformaldehyde

in organic material is approximately three weeks which is too short a period for hay preservation and Veloso et al (1974) concluded, that for the safety of the chicks, higher levels than 3% could not be used although this criterion does not apply to hay. There are however, economic arguments for not using such high levels of paraformaldehyde on hay.

iv) Glutaraldehyde



glutaraldehyde

Glutaraldehyde is a more effective bactericide than formaldehyde, but unlike formaldehyde its bacteriocidal activity is affected by p.H. (Rubbo et al 1967, Thomas & Russell (1974a) (1974b).

It is an extremely potent bactericide and bacterial sporicide and at a p.H of 8.0 it was four times more effective against Bacillus anthracis spores than at a p.H of 4.0 (Rubbo et al 1967). Unfortunately, as with formaldehyde, it is not so effective against fungi, although it does have fungicidal properties (Rubbo et al 1967).

This compound has potential as a hay preservative because being microbicidal, as opposed to microbistatic at low levels, it would kill most micro-organisms present in the hay. Unfortunately, as with formaldehyde it combines with organic material, rendering it inactive, although this means it would also become harmless to the animals eating the hay and their rumen flora. Glutaraldehyde is an

obnoxious compound and great care would be needed in handling it.

v) Gentian Violet

Gentian Violet is a common name for methylrosaniline chloride and a crude form of crystal violet. It has been added to animal feeds for several years, as a preservative, in the form of commercial products.

Chen and Day (1974) tested this compound, against seventeen other common anti-microbial agents, using the five fungal species Aspergillus flavus, A. fumigatus, Candida albicans, Fusarium moniliforme and Penicillium camemberti which are all common storage fungi. They found gentian violet was the most effective anti-microbial agent using a standard pour plate technique, with the exception of O - phenyl phenol which had similar inhibitory strength.

Crystal violet has been used for many years in chemical media to select for Gram negative bacteria, because it inhibits the growth of Gram positive bacteria at lower concentrations, being inhibitory to Gram positive bacteria at concentrations as low as 3 - 5 p.p.m and inhibitory to Gram negative bacteria at concentrations of approximately 50 p.p.m.

From this information, gentian violet appears to have a broad antimicrobial spectrum of activity, however, it has two major drawbacks as a hay preservative.

Firstly, it would probably kill or damage a significant proportion of the rumen flora. Secondly it is a strong purple dye which would

make handling extremely unpleasant.

There is neither information on the persistence of gentian violet on animal feeds, nor on its ability to resist microbial degradation and consequently its potential as a hay preservative cannot, at present, be fully evaluated.

vi) The Diols

Another group of potential hay preserving compounds is the series of linear, aliphatic 1,3 - diols and certain of their monoesters with the general formulae.

1,3 - Diols Diol esters

A patent, describing the use of these compounds as food preservatives and comparing them favourably with potassium sorbate and calcium propionate (Frankenfeld et al 1973), demonstrated the antimicrobial activity of these compounds. The potential of these compounds as grain preservatives was reviewed by the same workers in a later publication (Frankenfeld et al 1975).

A 1.3. - Monanediol (50%); 1,3 - butanediol 1 - monooctanoate (50%) mixture was compared with both 1,3 - heptanediol and propionic acid as a preservative for soy flakes at 100% relative humidity (Frankenfeld et al 1975). It was found the 1,3 - heptanediol inhibited mold growth most effectively, the remaining two preservatives

being similar in inhibitory activity. The rest of their work concerned 1,3 - Nonanediol (50%): 1,3 - butanediol - 1 - monooctanoate (50%) at various levels and with various relative humidities, on several different types of animal feeds. In all cases, levels of 0.5% and 1.0% of the mixture effectively delayed the onset of moulding.

Frankenfeld et al (1975) stated that further advantages of these compounds as preservatives were that they were non-toxic to rats, they could be metabolised by mammals as an energy source, they were odourless, non-corrosive and inexpensive.

Their main disadvantage as hay preservatives appear to be, difficulty in obtaining even application, being solids which are insoluble in water and they would therefore have to be dissolved in some suitable solvent, ethanol being used by Frankenfeld et al (1975). Also their persistance on hay is dubious because if they are readily metabolised by mammals they would almost certainly be readily metabolised by micro-organisms present in hay.

Their mode of action was unknown and whether they were microbicidal or microbistatic was not made clear, the results, however, suggested the latter.

How these compounds would affect the rumen flora is again an unknown quantity, but it appears unlikely they would remain active at high levelson hay for more than a few weeks due to microbial degradation, and would therefore be relatively harmless by the time the hay was eaten by the animals.

Finally, it was observed that the chain length of the diols had an effect on their preservation action, the most inhibitory compounds having 7 - 9 carbon atoms. Among the esters, the most effective molecules seemed to have a total carbon content in the range C_{10} to C_{15}. From this Frankenfeld et al (1975) concluded that the 1,3 - diols would make the best preservatives.

vii) The parabens

Esters of p - hydroxybenzoic acid, commonly known as the parabens, are another interesting group of compounds. The methyl, propyl and benzyl esters have been used as preservatives in pharmaceuticals for many years and are very effective at inhibiting the growth of micro-organisms, especially the bacteria (Wallhäuber 1974). 0.1% methyl parabens inhibited all microbial growth at p.H values of less than 7.0 and only 0.02% benzyl parabens was needed. These were higher concentrations than were required with formaldehyde and glutaraldehyde but lower than those needed for propionic and sorbic acids, in order to obtain microbial inhibition.

McRobbie and Parker (1974) reviewed the antifungal activity of the methyl to heptyl esters of p-hydroxybenzoic acid, against Aspergillus niger, using chemical agar media. They concluded that an increase in chain length caused a decrease in water solubility but an increase in their antifungal activity, as is the situation with volatile fatty acids.

The work of Crawford (1975), showed that between 10^4 and 10^6 aerobic sporeformers/g of soil were isolated in plate counts, which were capable of growing on methyl-hydroxybenzoate, and it therefore appears likely that large numbers of bacteria, also capable of degrading

hydroxybenzoates, would occur in hay.

As a conclusion, it appears the use of these compounds as hay preservatives is dubious, because not only would they probably be rapidly degraded but they are also insoluble in water making their application difficult. Results from this work show that they are, however, soluble in volatile fatty acids and since their activity is enhanced by a low p.H, they could be applied to hay as a solution in one of these acids, the components of this mixture, hopefully, acting synergistically against the hay microflora.

viii) Sodium nitrite

Sodium nitrite has been used in food preservation especially meats, and was thought to be worth considering as a hay preservative. Woolford (1975b) tested the antimicrobial activity of this compound against a range of silage micro-organisms. He found it had effective inhibitory properties which were enhanced by a lowering of the media p.H from 6.0 to 5.0. An excess of sodium nitrite in hay would almost certainly harm the rumen flora and possibly poison the animals, but it would have potential in hay preservation if used in relatively small quantities in conjunction with an organic acid.

ix) Other fungicides

Chemicals which are commonly used as fungicides for specific deterioration problems and others used to prevent disease formation on growing fruit, vegetables and cereals were given some consideration.

The majority of these chemicals are unsuitable for hay preserv-

ation for one or more of three reasons.

Firstly they are very specific, most of them being active towards only fungi or bacteria and usually to species within these groups. Compounds being so specific are unlikely to control the wide range of micro-flora which developes on mouldy hay.

Sypesteyn (1972) and Woodcock (1972) described a few of these fungicides, noting that one of the commonest, known as benomyl, showed a striking selectivity within the ascomycetes, whereas oomycetes, phycomycetes and bacteria were insensitive. Another example quoted was the oxathins which showed similar selectivity. In addition, Dennis (1975) demonstrated that both the fungicides Elvaron and Benlate were inactive against phycomycetes.

Secondly many of these compounds are toxic to mammals, and in the case of many storage treatments, the chemicals are applied and have to be then washed off after a short period or they would leave toxic residues. This method of preservation depends on the preservatives being microbicidal rather than microbistatic. Also chemicals sprayed on growing crops to prevent diseases are rarely applied during the few weeks preceeding harvest, so there would be sufficient time for the chemicals to be either washed off by rain or inactivated, examples of such compounds being thiram, borax and benzoic acid.

Thirdly many are too expensive. A chemical dip, which can be re-used, for preserving commodities including citrus fruits and carrots may be economical, but spray treatment on hay would not be so economical, examples being many anti-biotics and the fungicide benomyl.

Many of these compounds are reviewed by Smith (1972) and Marsh (1972).

There are a few of these treatments, however, which could be useful as hay preservatives including firstly sodium ortho-phenylphenate which was used to preserve fruits Smith (1972) and Marsh 1972) and carrots (Wells 1973). This compound has a broad spectrum of anti-microbial activity, is not toxic to mammals and is reasonably priced. Secondly, the fungicide captan which is relatively non-toxic to mammals and is effective as a pre-harvest treatment for fruit and vegetables Smith (1972) and Marsh (1972). Captan was shown to prevent fungal contamination of paddy seeds during four years storage (Kaul 1972).

X) Anhydrous ammonia and ammonium isobutyrate

Two compounds which have shown encouraging results in hay preservation are anhydrous ammonia (Knapp et al 1974) and ammonium isobutyrate (Yu Yu and Thomas 1975), these compounds are, however, considered in more detail later in this work.

C) The practical use of animal feed preservatives

i) The chemical preservation of animal feeds other than hay

The use of volatile fatty acids as animal feedstuff preservatives began to attract a lot of attention in Great Britain after BP chemicals published their patents (BP patents 1969a, 1969b 1971).

In their first patent they describe the preservation of animal feeds using propionic acid, their animal feedstuffs comprising of wheat, oats, rye, hay, maize, groundnuts, fishmeal, Soya beans,

tick beans, rape seed, sunflower seed and rice. Examples are quoted, including wheat containing 25% and 30% moisture levels being preserved for approximately 30 days by treatment with 0.5% propionic acid and oats at 20% and 30% moisture being kept mould free for nine months after treatment, also with 0.5% propionic acid. In the second patent it was recorded that binary and tertiary mixtures of formic, acetic and propionic acids were more effective preservatives than the acids used singly. In the third patent the range of feedstuffs was extended to include "animal protein feedstuff" examples being meat meal, blood meal, bone meal, feather meal, liver meal, liver and lung meal and other similar substances, thus showing the versatility of these acids as preservatives.

Since then, the use of volatile fatty acids to preserve animal fodder has rapidly expanded until a significant proportion of the 11 million tons of grain in Britain, used to feed our 13 million cattle and 8 million pigs, is now treated with propionic acid (Drysdale 1973).

Some of the many publications describing animal fodder preservation using various chemicals will be reviewed in this work.

One of the earliest of these publications was by Huitson (1968), which describes the basic advantages, of propionic acid grain preservation, over other methods, including dry storage, anaerobic storage and chilling. The acid treatment was the cheapest and was the only preservation effect that was maintained after the grain was removed from storage, other than expensive drying methods. This property facilitated handling and transport (Jones et al 1974).

Furthermore, the preservative action of propionic acid persists when treated grain is ground or rolled, thereby preserving the full nutritive value.

A graph was constructed which related the effective acid treatment levels to the moisture content of grain, and is shown in fig 1a, along with a similar graph produced by Sogn (1973). (fig 1b).

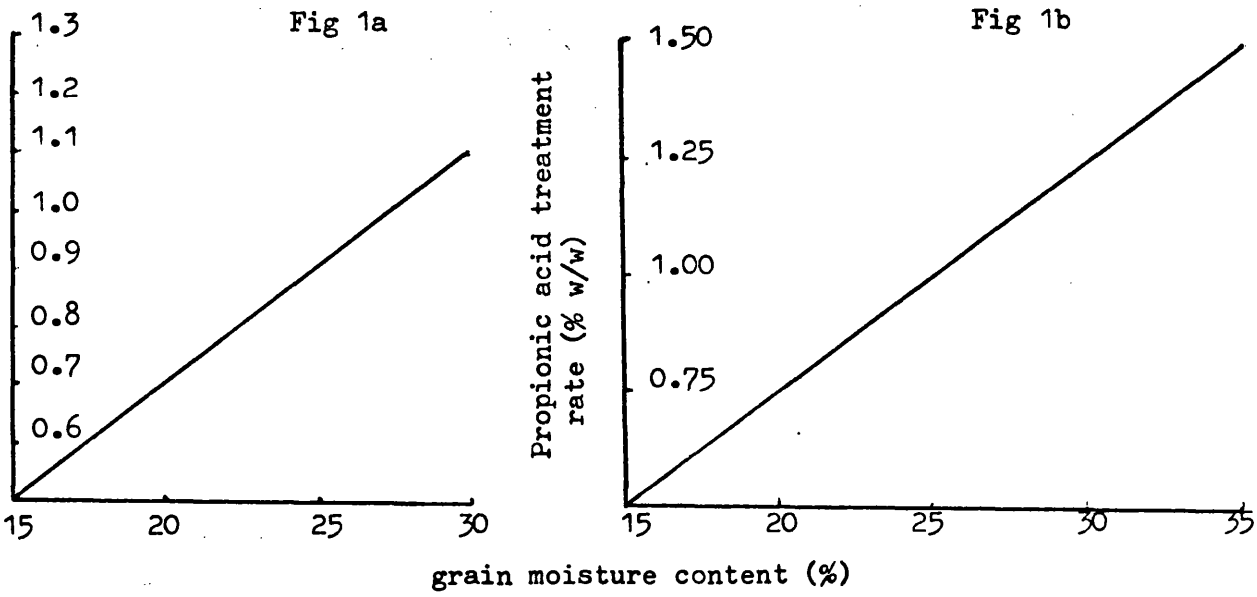


Fig 1. - The relationship between grain moisture content and propionic acid treatment rate Fig 1a from Huitson (1968) Fig 1b from Sogn (1973).

Huitson (1968) finally noted that propionic acid could only be used to preserve grain destined to be animal feed. Seed grain and barley for malting should not be acid treated as germination is inhibited, and propionic acid would have to be added to grain for human consumption at higher than permitted levels.

Bernier (1973) applied formic, acetic and propionic acids and mixtures of propionic and acetic acids to triticale, wheat, barley

and oats at 20%, 25% and 30% moisture, 100g samples of the grains being stored in stoppered bottles.

He concluded that barley needed the lowest levels of acid for preservation, oats required the most acid and that propionic acid and propionic, acetic acid mixtures gave the best protection. Treatments with 1% of these acids preserved 30% moisture grain for 14 months.

Ekström (1973) summarises his work using formic, acetic, propionic acids and a formic acid, formaldehyde mixture on moist fodder grains, in a table which is shown as table four.

Storage time in months	Acid	Per cent by weight of acid when the grain's moisture content is %.					
		25	27	29	31	33	35
6 - 8	Formic	1.30	1.40	1.50	1.60	1.70	1.80
6 - 8	Propionic	0.80	0.90	0.95	1.05	1.10	1.20
12	Propionic	1.00	1.10	1.20	1.30	1.40	1.50

Table 4. - The least amount of acid needed when treating moist grain - from Ekström (1973)

The use of acetic acid and the formic acid /formaldehyde mixtures were discarded because they gave relatively poor protection.

It was finally noted that with 25% moisture grain, acid preservation using propionic or formic acids was more economical than artificially drying the same grain, and less initial investment was needed.

Herting and Drury (1974a) tested, formic, acetic, propionic

n-butyric and iso-butyric acids and binary and tertiary mixtures, as grain preservatives. On corn, containing 20% moisture, they found acetic acid the least effective and iso-butyric the most effective single acids and the propionic acid: iso-butyric acid 1:1 mixture the most active preservative, the minimum inhibitory levels being shown as follows:-

acetic acid	1.06%
iso-butyric acid	0.56%
propionic acid	0.80%
iso-butyric acid: propionic acid 1:1	0.33%

In general treatments with acid mixtures were more effective than with single acids.

Herting et al (1974b) treated 20% moisture corn with fatty acids diluted with water. They found the more the acids were diluted the less acid was needed for preservation. In one experiment grain treated with 100% acid needed 0.34% acid treatment for preservation, however with a 50% water 50% acid mixture only 0.26% acid treatment was needed for preservation and with a 90% water 10% acid mixture only 0.17% acid gave control.

This was explained by assuming that volatile fatty acids are dimeric in the anhydrous state because of hydrogen bonds, however, water disrupts the acid to acid dimeric structure and yields water acid dimers. An excess of water could therefore have doubled the number of potentially active molecules.

Deyoe et al (1973) treated 25% moisture corn with acetic

propionic and sorbic acids, calcium propionate and a commercial formulation. The sorbic acid was dissolved in ethanol for application, the ethanol having an insignificant effect on mould inhibition. Their results, using grain stored in near anaerobic conditions in cans, showed 1.0% propionic acid treatment was more effective than 1.0% treatments with either acetic acid or calcium propionate and was as effective as the 0.1% sorbic acid treatment. When the grain was stored in cans and then aerated the 1.0% propionic acid treatment gave the best preservation being more effective than 0.5% sorbic acid, suggesting that sorbic acid was particularly effective in anaerobic conditions.

Bajinski et al (1973) recommended, as an animal fodder preservative, the use of sorbic acid : acetic acid mixtures in the proportions 0.05 - 0.2% : 0.2 - 0.8% along with an inert carrier which could have been white clay, bran or kieselguhr to name a few. Bajinski et al (1973) noted that sorbic and acetic acids acted synergistically which was not thought to be due to a lowering of the p.H because sorbic and propionic acids did not act synergistically. In one experiment, using winter barley grains, the untreated material moulded after 6 days, barley treated with 0.05% sorbic acid alone moulded after 8 days, barley treated with 0.30% acetic acid alone moulded after 13 days, whereas barley treated with both 0.05% sorbic acid and 0.30% acetic acid had not moulded after 32 days.

All these workers used the appearance of visible moulding as a sign that the preservative had ceased to work effectively and it was felt that more detailed examinations should have been employed because limited growth, especially of bacteria, could still have occurred. This could be an important point because, for example, harmful quantities of mycotoxins could be produced by limited fungal

growth.

Several workers have shown concern over possible harmful side-effects to animals consuming organic acid treated feeds, consequently they have carried out feeding trials using these feeds. Unfortunately, not all workers have been in complete agreement.

Huitson (1968) describes extensive trials which were carried out, feeding propionic acid treated grain to beef and dairy cattle, pigs, sheep and poultry and concluded that palatability of the grain was not impaired, feed conversions and growth rates were as good or better than untreated grain and the livestock showed no adverse effects.

Stajanovic et al (1972) fed naturally and artificially dried corn and propionic acid preserved corn to albino rats and concluded that the acid preserved corn had considerably better biochemical and micro-biological characteristics and that feed consumption and utilisation, body weight gain and nitrogen retention were all increased using acid preserved feed.

Jones et al (1970) noted that pigs would tolerate 8% organic acid on feed, that the acid treatment improved its digestibility and that cattle were unaffected by fodder treated with 4% propionic acid.

Clark et al (1973) concluded that moist grain treated with propionic acid had 10% better nutritional value for cattle and pigs than the same grain which had been dried. In contrast Marion et al (1973) noted that 6% propionic acid or 6% acetic acid on beef cattle fodder did slightly reduce daily weight gains of cattle but that 4% acetic acid increased their growth rate while 4% propionic acid had no effect. Furthermore the 6% acid treatments depressed feed intake

whereas the 4% treatments had no effect and feed containing 4% and 6% acetic acid increased the rumen acetate while 6% propionic acid treated feed highly significantly increased the rumen propionate and decreased its butyrate.

Ekström (1973) fed pigs and baby beef with untreated and acid treated barley. The growing pigs fed on acid treated grain showed poorer weight gains and feed conversions than the controls, however, with the baby - beef, the formic and propionic acid treated grain gave better daily increases than the controls.

The work done concerning the volatile fatty acid preservation of animal feeds draws several basic conclusions.

Firstly, that the higher the moisture content of the feed the more acid would be needed.

Secondly, the treatments work effectively if sufficient preservative is applied and applied evenly.

Thirdly, as long as practical levels of acid are used, it seems unlikely there will be any harmful effects on the animals eating the feeds.

Fourthly, mixtures of the acids are more effective than acids alone, acetic acid appears the least efficient preservative and in general the larger the acid molecules the more strongly anti-microbial they become.

Finally, acid treatment of moist grain is presently the most

economical method of preserving it.

There is a possibility that in a few cases volatile fatty acids, added to animal feeds, could help to cure infections already in the animal's body, for example Wood (1968 and 1969) showed that formic acid, added to partridge food, could act as a prophylactic for moniliasis, a disease caused by the yeast Candida albicans.

As an aside, it is of interest to note that propionic and acetic acids have even been tested as tobacco preservatives (Lucas et al 1973), propionic acid preserving tobacco, which was stored in flasks, more effectively than acetic acid.

Other chemical methods of grain preservation have been tried. Majunder et al (1973) experimented with the use of gaseous fumigants including sulphur dioxide, ethylene oxide, ammonia and methyl bromide. They concluded that these products showed promise, however, they were concerned that some of them may react with chemical components of grain for example ethylene oxide could react to form chlorohydrins. Other fumigants, including crotyl bromide and formaldehyde, are known to denature proteins. Therefore more work obviously needs to be done in this area.

The problems of fumigating hay are greater than with grain, since the latter is stored in silos whereas the former is usually kept in open barns. Also, it would be easier to force gases through grain than through bales of hay especially as grain is often dried using an air flow and the fumigants could be incorporated in this system. However, ammonia injection into hay (Knapp et al 1974) has given encouraging results, and fumigation could prevent hay from moulding.

ii) The chemical preservation of hay

Unfortunately there is not as much available data on the chemical preservation of hay, as with grain, and the results which have been obtained are less encouraging, but as with grain, volatile fatty acids have been most commonly used.

Candlish et al (1973) tested formic, acetic and propionic acids as hay preservatives, along with a commercial product which was a mixture of propionic and acetic acids, using three different types of hay. In one experiment the treated and untreated hays had a moisture content of 40%. The untreated bales heated to 58°C and moulded visibly after three days, hay treated with 0.1% formic acid heated to 39°C and showed only traces of mould whereas the commercially treated hay heated to 33°C and showed no visible signs of mould, whereas the acetic acid treatment appeared to have encouraged mould growth.

Drew et al (1974) reported that alfafa hay needed chemically preserving when the moisture content was 22% or higher, and that for the hay to be considered preserved the temperature should not exceed 38°C. They applied an undefined preservative at a rate of 0.1% to hay containing 25% moisture. The untreated hay heated to 42°C whereas the treated bales only heated to 36°C suggesting a measure of control. The same hay, at 30% moisture, treated with 0.1% preservative, heated to 38°C suggesting some moulding had occurred. The actual chemical composition of their preservative was not given, however, from their comments it seems likely it was propionic acid, with or without acetic acid.

The fact that the treated 25% moisture hay heated could have been due to the activity of grass enzymes which are capable of raising hay temperatures (Gregory et al 1963b). However, this seems unlikely because organic acids inhibit this activity. Overall this paper was unusually encouraging as regards the effectiveness of chemical hay preservatives.

A more scathing report on propionic acid hay preservation was produced by Benham et al (1975). They carried out three separate trials using an acid applicator which consisted of several nozzles on the face of the ram in the pick-up baler.

In the first trial they used hay at 29% moisture which was treated with 0.0%, 0.5% and 2.5% propionic acid. The 0.5% treatment gave the best control of heating whereas the 2.5% acid controlled the growth of thermophilic and thermotolerant moulds most effectively. Micro-organisms which were classed as "Farmer's lung" organisms, presumably they were thermophilic actinomycetes, did not increase in any of these hays.

The second trial used hay at 41 - 46% moisture, treated with 0.0%, 0.7%, 1.4% and 2.0% propionic acid. A delay, but no significant reduction in heating by the acid treatments was recorded, the 2.0% treated hay heating to 53°C. In all four treatments the moulds and "Farmer's lung" organisms increased considerably.

The third trial consisted of hay at 28% moisture, with 0.0% and 2.0% propionic acid levels being applied. When they were stored in piles, the temperature of the control bales rose to 55°C whereas the acid treated bales heated to only 37°C thus showing some control of

heating. With single bales surrounded by polystyrene chips the untreated bale heated to 47°C whereas the treated bale did not heat above 30°C. A microbiological examination of the single bales revealed that the increase in mould and "Farmer's lung" organisms had been controlled but not prevented by the acid.

Chemical analyses on these hays, were carried out, and they revealed few biochemical changes due to the acid treatments, except for a slight reduction in digestible organic matter, although the authors did not discuss this point. These findings tend to contradict the findings of Candlish et al (1973) who undertook some hay feeding trials, using sheep, and concluded that the dry matter intake was highest for the untreated hay, which suggested that the digestibility was lower as the two are usually inversely related. The formic acid treated hay, when fed to sheep, caused a loss in weight, because the sheep would not eat enough, possibly due to the acid making the hay unpleasant to the animals.

These results, especially those of Benham et al (1975), show that the organic acid preservation of hay is more difficult than in the case of grain. Using present techniques, higher levels of acid are needed for hay preservation than for grain at the same moisture levels, as can be seen by comparing these results with the graphs of Huitson (1968) and Sogn (1973) as shown in Fig.1 (page 56)

As a possible alternative to volatile fatty acids, Knapp et al (1974) experimented with anhydrous ammonia as a hay preservative and compared it with propionic acid. They found that both 1.0% anhydrous ammonia and 1.0% propionic acid treatments reduced heating and dry matter loss, when compared with untreated hay, and prevented

the formation of visible moulding. There appeared to be little difference between the two treatments, however, the propionic acid was added to the hay by pouring an aqueous solution as evenly as possible on the cut edge of each bale, and this was unlikely to have been as effective a method of application as spraying. The ammonia was applied through a hose placed between the lower bales, of piles of bales, which had been covered with polyethylene and it appears probable that this application method could also have been improved, possibly by injecting the gas into the middle of the bales.

It is difficult to assess the comparative values of ammonia and propionic acid as hay preservatives from this work because of the poor application methods used, however, one interesting fact noted was that a 1.67% ammonia treatment of hay which had heated to 63°C immediately caused the temperature to fall and it was reduced to 35°C after 48 hours. The point was made that ammonia could be used to protect moist hay against the danger of spontaneous combustion.

Yu Yu and Thomas (1975) compared propionic acid and ammonium isobutyrate as alfalfa haylage preservatives and concluded that both chemicals, at the same levels, were equally effective in reducing heat developement, total fungal counts and losses in nitrogen utilisation for the haylage during storage.

Anhydrous ammonia and ammonium isobutyrate could both be alternatives to volatile fatty acids as hay preservatives, and they have the advantages of being less unpleasant to handle and would not corrode machinery.

d) Problems concerning the chemical preservation of hay

The preservation of hay using chemical methods, which has been reported in the literature, is generally disappointing, with very few treatments giving complete control of deterioration.

In chemically defined media, which are better growth substrates than even very moist hay, less than 1.0% propionic acid, at the p.H of freshly baled hay, would totally inhibit the growth of all micro-organisms commonly found in hay,as can be seen from results obtained during this work and which are described later. Why therefore, do higher levels of acid fail to preserve damp hay?

There are three possible explanations. Firstly, poor distribution of preservative, secondly, microbial degradation of the preservative and thirdly, reactions of the preservative with chemical components of hay. Considering the third point, there appear to be few compounds in hay which would react with volatile fatty acids rendering them inactive and they are unlikely to be neutralised because fresh hay is weakly acidic. Possible chemical reactions of hay with other preservatives have previously been discussed and therefore only the first two points will be considered in more detail.

i) Poor distribution of preservative

The eveness of distribution of a preservative within a bale of hay will be a major factor determining the effectiveness of that preservative. One possible way of overcoming the problem would be to use a compound volatile enough to be able to permeate through the hay, however, there is the potential danger of it seeping out into

the atmosphere and being lost.

The work of Lacey and Hill (1969) demonstrates how important an even application of propionic acid on hay is, for it to give complete protection.

They filled dewar flasks with three layers of damp hay, the middle layers being treated with 2%, 4% and 10% propionic acid. After storage they examined the hay and noted that the 2% treated hay had been invaded by moulds, the 4% treated hay had little moulding whereas the 10% treated hay had prevented moulding only 5 cms into the adjacent untreated hay. These results suggest the movement of propionic acid through hay is very limited and that such movement as occurs, would have little beneficial preserving effect, unless the application rate was considerably higher than could be used in practice.

Similar results were obtained by Smith and Stevenson (1975) using propionic acid treated corn in unsealed laboratory silos. They concluded that condensed water movement lowered the propionic acid to sub-inhibitory levels on the treated corn.

In order to assess the importance of this problem, the distribution of the preservative within treated hay bales has firstly to be assessed and there are two techniques available for doing this.

Firstly, using spray tracers. The theory behind this method is to spray a dye on the hay using a preservative applicator and then to quantitatively assess the coverage by extracting the dye deposit and measuring it colourimetrically or to visually assess the coverage.

Strong concentrations of highly coloured dyes such as nigrosin have been used, but more recently, fluorescent materials have replaced them, because they can be measured two hundred times more sensitively than conventional dyes.

A fluorescent dye is one which absorbs radiation at a certain wavelength and re-emits it as light of a greater wavelength. The dyes usually used in this technique absorb long wave ultra-violet light (around 365 nm) and re-emit visible light (400 - 700 nm).

When hay is viewed under ultra-violet light, it has a natural blue and yellow fluorescence and therefore dyes which fluoresce red or green would be most clearly visible on hay.

The use of fluorescent dyes to measure spray deposits is reviewed by Sharp (1974). He classifies them as water-soluble dyes, oil-soluble dyes and pigments.

Water soluble dyes are the easiest to use, being simply dissolved in water at concentrations of approximately 0.1%. along with an inert carrier, examples being starch or polyvinyl alcohol, which reduce quenching of the fluorescence caused mainly by the chlorophyll in the grass. The main disadvantages of these dyes are that they fade when exposed to ultra-violet light and sometimes heat, and they are either non-fluorescent or only weakly so when dry.

Fluorescent pigments are generally a more suitable group of compounds, mainly because they show up more clearly when sprayed on

hay and viewed under an ultra-violet lamp, and also they do not fade so rapidly, as the water soluble dyes. These materials are fluorescent dyes absorbed and sealed within a resin structure, and they provide a very stable high -intensity fluorescent material. They can be obtained, from Haeffner and Co. Ltd., as particles ranging in size from 1-9 μm in diameter, but for spray assessment on hay a group having 1-2 μm diameter particles are used. These compounds are available in the colours neon red, fire orange, arc chrome and saturn yellow. Saturn yellow would tend to be masked by the natural yellow fluorescence of hay, however, the remaining three colours would be suitable.

Sharp (1974) describes a fluorimeter which can give quantitative results with these dyes, being able to detect 3×10^{-9} g of saturn yellow and accurately measure 10×10^{-9}g.

Fortunately these compounds have very low mammalian toxicity and therefore the treated hay could be fed to animals if it was required.

The main disadvantage of using fluorescent dyes to assess spray coverage is that they do not fluoresce in acids (Sharp 1974) and therefore would have to be sprayed on hay dissolved in another solvent, usually water, however, water containing these dyes is unlikely to have the same physical properties including surface tension and viscosity, as the hay preservatives and would therefore give a different spray pattern. The general pattern of preservative coverage on hay using a particular applicator could be assessed using this technique and possibly even of more importance, different application methods could be compared.

The second method is to assess directly the preservative level on the hay immediately after spraying. There appears to be little published data on estimating volatile fatty acids applied to foliage, the most common practice appearing to be an extraction of the acid from the material using dilute sulphuric acid and then an estimation of the organic acid by gas liquid chromatography. A more promising technique appears to be to heat the hay and organic acid with a small container of caustic soda solution and again to estimate the organic acid in the solution, after acidification, by gas liquid chromatography. A suitable gas liquid chromatographic technique based on one by (Huysteen 1970) is described later in this report.

Benham et al (1975) tried to estimate the propionic acid on treated hay samples but obtained very low percentage recoveries, rarely exceeding 50% of the applied acid, and usually only 20 - 30%. Unfortunately they did not describe the method they used, but they noted that they were uncertain where the error lay.

The majority of preservatives applied to hay could be assessed by some method and such techniques would give a general picture of preservative distribution within a bale, however, they would not demonstrate the pattern of application on single strands of hay as would the fluorescent dye methods. To conclude, it appears that the use of both methods is to be recommended.

ii) Preservative degradation by micro-organisms

With materials like hay which are often stored for periods of

several months and which contain a diverse range of micro-organisms, the growth of organisms, which are able to degrade the preservative added, is likely to occur, and in some cases the unwanted situation may arise where a preservative could encourage the growth of micro-organisms, as has been observed in the case of acetic acid on hay (Candlish et al (1973)) and organic acids in general in low quantities on animal feeds (B.P. patents 1969a and 1969b), by being utilised as a nutrient source.

It is beyond the scope of this report to discuss the microbial degradation of every potential hay preservative, however, a few will be briefly discussed particular attention being paid to the volatile fatty acids.

Formaldehyde - There are few micro-organisms which have been recorded as being able to degrade formaldehyde, however, Cooney and Levine (1972) reported that many methane and methanol utilising bacteria can degrade formaldehyde by incorporating it in the following pathway.

$$\underset{\text{methane}}{CH_4} \longrightarrow \underset{\text{methanol}}{CH_3OH} \longrightarrow \underset{\text{formaldehyde}}{HCHO} \longrightarrow \underset{\text{formic acid}}{HCOOH} \longrightarrow \underset{\text{end products}}{CO_2+H_2O}$$

They also recorded some yeasts that similarly metabolised methanol. Sakaguchi et al (1975) reported fungal species which could grow in chemical media containing 0.1 to 0.2% formaldehyde and which could also degrade the chemical. One of these fungi (Paecilomyces varioti) is the same species as occurs commonly in hay.

Organic acids - One of the advantages of preserving hay with certain

organic acids, is that they are non-toxic to mammals and that they can actually be utilised by the mammalian body as an energy source and hence increase the hay's nutritional value. Unfortunately many micro-organisms are also capable of degrading these acids. It is probable that in an acid treated bale of hay there will be patches of hay with little or no preservative and in these areas micro-organisms would start to grow and some of them would spread into the treated hay and breakdown the acids present, which would then permit the growth of further organisms.

Volatile fatty acids would probably be degraded by one of the mechanisms described in the following section.

Callely and Lloyd (1964) reviewed the metabolism of propionate and described the five major pathways used before it was incorporated into the tricarboxylic acid cycle.

a) The succinate pathway - This is the main pathway used by mammalian cells, where the propionate is activated, then undergoes carboxylation to propionyl CoA, then to methylmalonyl CoA which is isomerised to succinyl CoA; this latter reaction requires vitamin B_{12} as a co-enzyme. This pathway is also used by some photo-synthetic bacteria (Murayama and Kitamura 1975).

b) Acrylate pathway - Propionate is oxidised to acrylate then to lactate and finally to pyruvate.

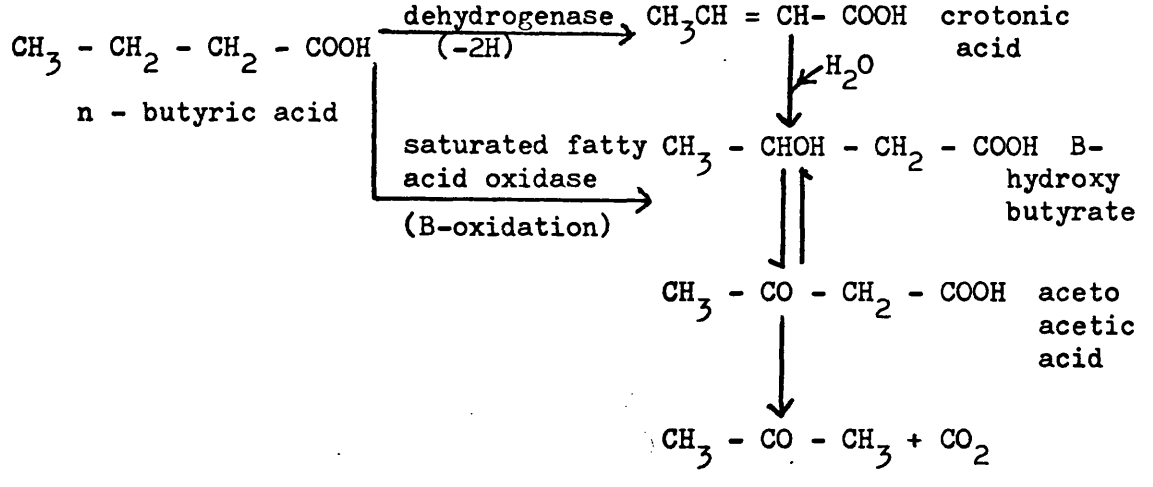
c) Malonic semialdehyde pathway (Fig 2) - Propionate is converted to propionyl - CoA then to acrylyl - CoA then to B-hydroxy-propionyl

CoA then to free β-hydroxypropionate and finally to malonic semi-aldehyde which is oxidised to acetyl CoA and CO_2.

d) The malonyl - semialdehyde - CoA pathway - This pathway is basically the same as pathway C except that B- hydroxypropionyl CoA is oxidised to malonyl - semialdehyde - CoA then converted to malonyl - CoA from which an acetyl unit is formed.

e) α - hydroxyglutarate pathway - The propionate goes to propionyl CoA which condenses with glyoxylate to give α - hydroxyglutarate which is cleaved to give acetate and lactate, the lactate going to pyruvate. This is the probable pathway used by Eshcherichia coli which has been adapted to use propionate as its sole carbon source.

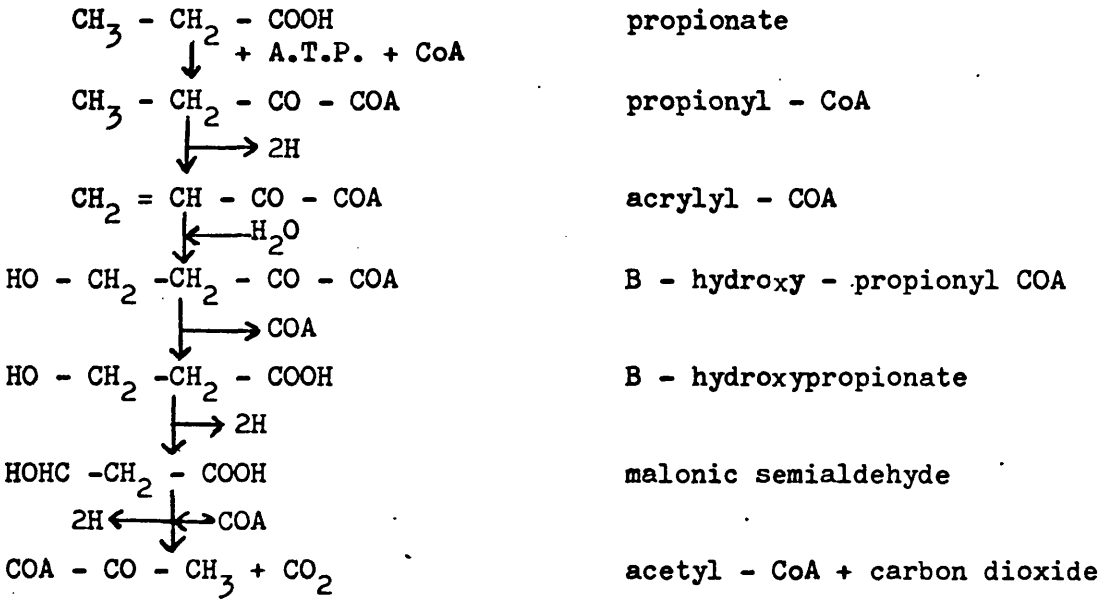
Mukherjee (1952) studied the metabolism of n-butyric acid by the mould Aspergillus niger and concluded it was degraded by the following mechanism.



This pathway is similar to the malonic semialdehyde pathway previously described (Callely and Lloyd 1964) except for the final stage. Mukherjee (1952) thought that the n-butyric acid could be

converted to B-hydroxybutyrate either directly by β-oxidation or by being oxidised to crotonic acid which was then hydrated.

Fig 2 - The malonic semialdehyde pathway for propionate utilisation (From Callely and Lloyd 1964)



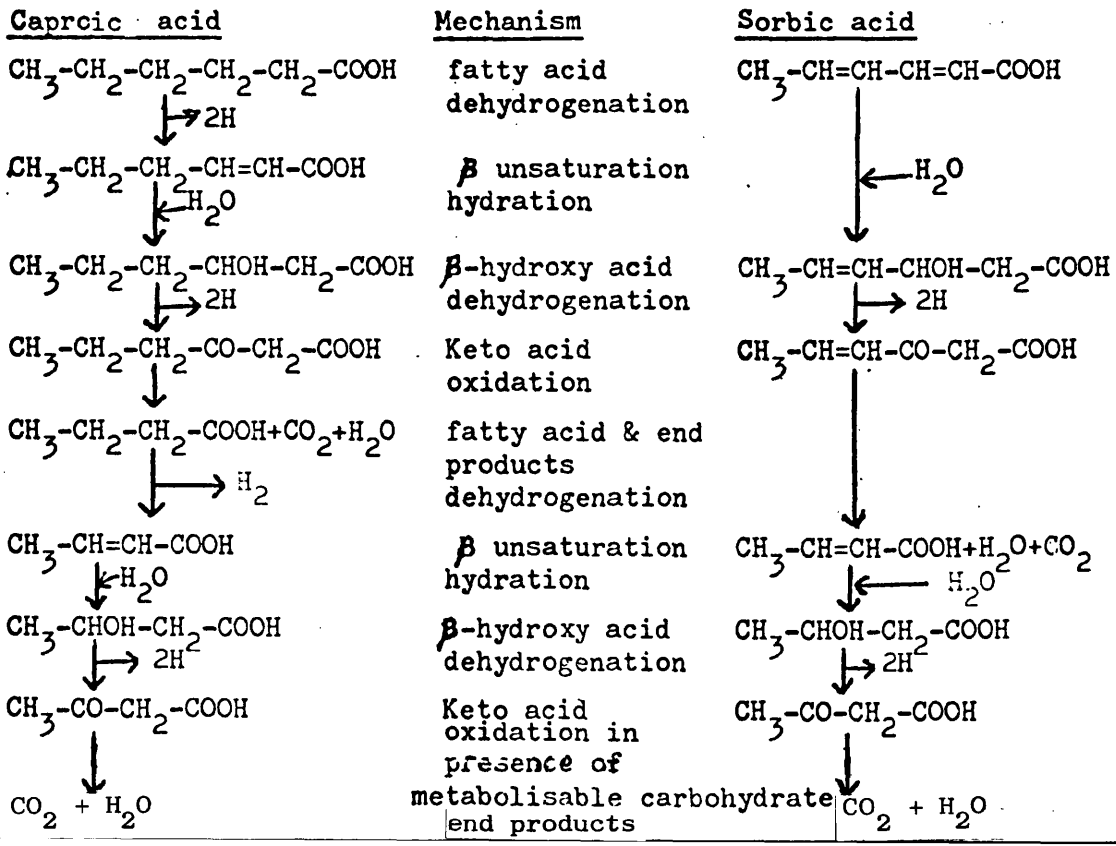
Melnick et al (1954) reviewed saturated and unsaturated fatty acid metabolism by moulds. They concluded that the pathway devised by Mukhergee (1952), applied generally to the degradation of saturated fatty acids.

Sorbic acid is the unsaturated molecule of the naturally occurring fatty acid caproic acid and is metabolised by a similar mechanism involving the Wieland dehydrogenation - hydration - dehydrogenation scheme. Saturated fatty acid degradation was summarised and compared with sorbic acid metabolism, as shown in fig 3, by Melnick et al (1954), all the steps in this scheme being reversible and the acids occurring as esters of coenzyme A.

From their own work and that of Mukherjee (1952), Melnick et

al (1954) concluded that the inhibitory action of sorbic acid on mould growth is due to the fact that it inhibits the dehydrogenase system because it creates an excess of the end-product of the dehydrogenase reaction. These dehydrogenase enzymes are so basic to cell metabolism that mould growth is inhibited provided there is little mould present, if however, the mould population is high, the concentration of the enzyme systems is increased and sorbic acid is then metabolised. When considering the safety of using sorbic acid on animal feeds it was concluded that it would be impossible to administer sufficient of the acid to inhibit the dehydrogenase enzyme systems in the mammalian body and therefore sorbic acid would make a very safe preservative. The increase in the anti-fungal activity of sorbic acid which occurs with a decrease in p.H was thought to be because the undissociated acid could pass through the cell membrane more readily.

Fig 3 - Normal metabolic degradation of fatty acids by moulds and by the animal organism (Melnick et al 1954)



Two carbon fragments enter Kreb's citric acid cycle and are metabolised to CO_2 and H_2O.

The later works of Kurogochi et al (1974 and 1975) suggested that a different pathway was used for the degradation of sorbic acid by a Mucor spp. They proposed that the fungus reduced the carboxyl group to an alcohol group giving an β unsaturated alcohol which was further reduced to give an β saturated alcohol and the saturated alcohol was then converted to trans-4-hexenol.

It appears that the organic acids which have been considered as hay preservatives can be degraded by various metabolic pathways and that many micro-organisms, several of which almost certainly occur in hay, possess one or more of these pathways. It is logical to assume, therefore, that these preservatives need to be applied to hay so that microbial activity is totally inhibited, if not, then preservative degradation would almost certainly occur.

It is of interest to note that some of these enzymic systems occur in the mammalian body and this would help to account for the low toxicity of these organic acids.

SECTION C - MICRO-ORGANISMS INVOLVED IN THE DETERIORATION OF ANIMAL FEEDS IN STORAGE

a) The fungi

There are a large number of fungal species involved in the biodeterioration of stored animal feeds, including hay.

An important characteristic of moulding hay is that it heats, and since the majority of fungi cannot grow above 30°C, then a relatively specialised group of heat tolerant or heat requiring fungi grow in moulding hay, therefore this work will concentrate on this particular group of fungi. Most of this discussion applies to other self-heating organic materials, although different fungal species may be involved.

Fungi which grow at high temperatures can be divided into two groups as defined by Cooney and Emerson (1964). Firstly there are the thermophilic fungi which have a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C. Secondly there are the thermotolerant fungi which have a maximum growth temperature near 50°C but a minimum growth temperature well below 20°C, Cooney and Emerson (1964) review the thermophilic fungi and describe the important role they play in the degradation of self-heating organic material.

Fungi, in these groups of moulds, which have been isolated from self-heating hay include:-

Thermophilic fungi:- Mucor pusillus, Mucor miehei, Talaromyces

dupontii, Thermoascus aurantiacus, Chaetomium thermophile var caprophile, Thermomyces lanuginosa, T. stellata and Malbranchea pulchella var sulfurea.

Thermotolerant fungi - Aspergillus fumigatus and Absidia ramosa.

The occurrence of thermophilic and thermotolerant fungi is widespread. Evans (1972) studied their spore numbers in air and found Aspergillus fumigatus the commonest with Thermomyces lanuginosa, Absidia corymbifera, A ramosa and Mucor pusillus all occurring frequently. The spore counts varied throughout the year, rising steadily from March to August then decreasing to October and rising again reaching a maximum in January. No explanation for this pattern was given.

Thermophilic and thermotolerant fungi also occur commonly in soil. Alpinis (1963) isolated Mucor pusillus, Talaromyces dupontii, Thermoascus aurantiacus, Thermomyces lanuginosa, Absidia ramosa, Aspergillus fumigatus and Sporotrichum thermophile from various soils. A. fumigatus again was the most frequently occurring of these fungi. All of these moulds only occurred in the top soil layers, especially where organic material was plentiful, probably due to favourable conditions of aeration, moisture and high soil temperatures in the summer. Eggins et al (1969 and 1972) isolated several more thermophilic and thermotolerant fungi from soil and found them growing actively in soil exposed to sunlight but not in the shade.

Species of these fungi have been found in large numbers in self-heated mushroom compost (Fergus 1964), in peat (Kuster and Locci 1964), in wheat straw compost (Chang and Hudson 1967a), they play a part

in wood degradation (Ofosu-asiedu and Smith 1973) and of course they occur in hay (Gregory and Lacey 1963a, Gregory et al 1963b).

It can be seen that their occurrence is widespread and they will grow actively in many environments providing the temperature rises well above 20°C.

Cooney and Emerson (1964) and more recently Chapman (1974) and Rosenberg (1975) have studied the temperature requirements of these micro-organisms. They have temperature optima, for growth, of about 40°C - 45°C the most thermophilic species being Thermomyces lanuginosa with a growth range of 30°C - 60°C, however, most of these fungi will grow at 55°C, and could therefore, play an important part in the self-heating of hay, up to temperatures of 55°C - 60°C.

Rosenberg (1975) demonstrated that most of these fungi have p.H optima of approximately 7.0, however, Mucor pusillus M. meihei and Aspergillus fumigatus preferred slightly acid conditions.

The important role that these fungi play in the deterioration of hay appears to be partly due to the strong cellulolytic activity that some species have shown. Fergus (1969) and Tansey (1971) have made studies in this field and they have shown that Chaetomium thermophile and Aspergillus fumigatus can degrade native cellulose, Malbranchea pulchella var sulfurea can degrade carboxymethyl cellulose whereas Mucor pusillus, M. miehei T. lanuginosa T. stellata and Thermoascus aurantiacus are non-cellulolytic.

Considering these results and the microbiological studies of

Gregory et al (1963b) and Chang (1967b) it appears that there are similar successions, in the growth of these fungi, in moulding hay and wheat straw, and these appear largely to depend on temperature and p.H changes and utilisation of various carbon sources. A possible explanation for these fungal successions is described below.

As the temperature of the material rises above 30°C the mesophilic fungi stop growing and the thermophilic and thermotolerant species take over. Mucor pusillus and Absidia ramosa, which are very rapidly growing and sporulating phycomycetes, liking the slightly acid conditions of freshly baled hay, become dominant at first, utilising the available sugars. When the sugar supply is exhausted, these fungi cannot grow and the cellulolytic Aspergillus fumigatus, Chaetomium thermophile and Malbranchea pulchella var sulfurea become dominant. As they produce cellulases, the sugars reappear and secondary invading fungi including Thermomyces lanuginosa, T. stellata and Thermoascus aurantiacus proliferate. The phycomycetes do not usually reappear, possibly because the p.H of the material has become too high, which is more suitable for the other fungi, and the temperature also may be too high, the phycomycetes not being as thermophilic as the other moulds.

Another important feature of the thermophilic and thermotolerant fungi, is their strong implication in many human and animal diseases. This topic has been discussed previously, especially considering the fungi Aspergillus fumigatus and Absidia ramosa, whose potential hazards cannot be over emphasized (Austwick 1963).

The ability of these fungi to grow at high temperatures has

not been satisfactorily explained. Crisan (1973) put forward four hypotheses which were (a) lipid solubilisation, (b) rapid resynthesis of essential metabolites, (c) Macromolecular stability and (d) ultra-structural stability. Only the latter two hypotheses are now considered to be of importance. Gamson et al (1975) observed one to several large membrane bound inclusion bodies in the cytoplasm of Thermomyces lanuginosa, T. stellata and Humicola insolens var thermactea, but none were observed in the hyphae of Chaetomium thermophile var caprophile, Mucor pusillus or Thermoascus aurantiacus. The results were not discussed in detail and no firm conclusions were drawn, leaving the explanation still unresolved.

b) The Actinomycetes

The actinomycetes are a group of micro-organisms which occur in large numbers in self-heated material. If the material has heated considerably then the famillies Thermoactinomyces, Thermomonospora and Micropolyspora are usually abundant, however, Streptomyces spp usually predominate in organic material which has heated little. Self-heating environments from which the actinomycetes have been studied include mushroom compost (Fergus 1964), wheat sraw compost (Chang and Hudson 1967a) and hay (Gregory and Lacey 1963a).

The classification of actinomycetes is not as well studied as with the fungi and bacteria. The works of Lechevalier and Lechevalier (1967) and Cross and Goodfellow (1973), describe the important families of the Actinomycetales and the classification of the Streptomycetes was greatly clarified in the International Streptomyces Project (Shirling and Gottlieb 1966, 1968a, 1968b, 1969 and 1972) which was summarised into working keys by Kuster (1972) using criteria including melanin production,

pigment production, sporophore and spore properties, carbohydrate utilisation and mycelial colour.

Material, including hay, which has heated, contains thermophilic actinomycetes. This specialised group of actinomycetes, has been studied by Henssen and Eberhard (1967) and Cross 1968) and includes the species Thermoactinomyces vulgaris formerly known as Micromonospora vulgaris (Cross and MaCiver 1968) and Micropolyspora faeni formerly known as Thermopolyspora polyspora (Cross and MaCiver 1968) which are considered to be the micro-organisms mainly responsible for 'Farmer's lung'. The actinomycetes which occur in mouldy hay have been described by Corbaz et al (1963).

The role actinomycetes play in hay biodeterioration is not very clear. Thermoactinomyces vulgaris and Micropolyspora faeni are non - cellulolytic, however, Streptomyces thermoviolaceus produces cellulases (Fergus 1969), as does Thermomonospora curvata (Stutzenberger 1971, 1972) and in the latter studies, most of the cellulases present in a municipal solid waste compost were found to be produced by this actinomycete species. Both of these cellulolytic actinomycetes have been found in hay, and may play an important part in breaking down hay cellulose, especially at high temperatures of between 40°C and 60°C.

Some species of thermophilic actinomycetes can grow at higher temperatures than the thermophilic fungi, a few Thermoactinomyces vulgaris strains being able to grow at 63°C (Flockton and Cross 1975). Tendler and Burkholder (1961) reported actinomycetes which would grow at 67°C. Therefore these micro-organisms, along with thermophilic bacterial species, could raise hay temperatures to nearly 70°C.

A feature which makes actinomycetes particularly interesting in

this work, is their apparent resistance to volatile fatty acids, including propionic acid. The addition of calcium or sodium propionates to chemical media has been recommended for actinomycete isolation (Durbin 1961, Crook et al 1950), therefore it could be the inhibition of this group of micro-organisms which determines the level of organic acid to be applied to damp hay to prevent it from deteriorating.

c) The bacteria

Little work has been done on the bacteria associated with hay biodeterioration.

In general, bacteria require a higher water activity than fungi for growth (Scott 1957, Ingram 1957), and probably only become important in the moulding of wetter hays.

Gregory et al (1963b) carried out bacterial counts on hay, and found their numbers rose rapidly in damper hays after baling. Little was done on the identification of these organisms except that Lactobacilli spp were present as a small proportion of the total bacterial population and that after a period of storage Bacillus licheniformis became dominant. Festenstein et al (1965) found Micrococus sub-group 6 was common in moulding hay stored in dewar flasks.

Druce and Thomas (1970) studied psychrotrophic bacteria in hay and stated that they often constituted more than 50% of the total bacterial flora in grass and newly baled hay, but were less important in older hays.

Fluoresent Pseudomonads were dominant, but Acinetobacter, Micrococci Coryneforms and members of the Coli-aerogenes group were all present.

Despite this work of Druce and Thomas (1970), as with fungi and actinomycetes, it is the thermophilic bacterial species which are important when considering the deterioration of hay. Allen (1953) reviewed the thermophilic aerobic bacteria, noting that some species were capable of growing at 70°C and above, and that they occurred widely in nature including in soil and in hay. He concluded that thermophilic strains of almost any mesophilic Bacillus spp could occur, and that this feature could well apply to other bacterial species. More recently Brock and Freeze (1969) described a Gram -ve bacterium which could also grow at 70°C.

The importance of bacteria in hay deterioration is uncertain. They can reach numbers of 10^9/g (Gregory et al 1963b) in wetter hays and at this level they must play a significant role in hay deterioration. Allen (1953) noted that some thermophilic Bacillus spp produce cellulases and Lee and Blackburn (1975) described the rapid degradation of filter paper by a thermophilic Clostridium spp, which was considered to be typical of many similar strains isolated from hay.

The bacteria which occur in deteriorated hay, have not so far been linked with the diseases, to both man and animals, which have been attributed to the mouldy fodder. There has been little published information in this field, but since many bacteria are known to produce toxins in food, for example the enterotoxin of Staphylococus aureus, and pathogenic bacteria are widespread in nature, it is by no means certain that the bacteria in deteriorated hay do not constitute a serious health hazard.

d) The anaerobic growth of micro-organisms

When moist hay or any other organic material is stored in bulk, plant and microbial respiration will take place. This activity will rapidly utilise most of the oxygen present within the material, especially towards the middle of the bulk, and will replace it with carbon dioxide, so creating anaerobic or near anaerobic conditions. These conditions select for the growth of certain anaerobic micro-organisms including Lactobacillus and Streptococcus species of bacteria, which have been used as a means of preserving grass, in the form of silage, with considerable success.

Gregory et al (1963b) described a typical hay stack, and noted that in the central region the hay was brown and acidic and contained few fungal and actinomycete spores but many bacteria. The outer layers of the stack contained hay microbiologically similar to that found in bales. This acid brown hay was a result of strictly anaerobic conditions, which permitted the growth of bacteria which produced acids including lactic and acetic.

This acid brown hay does not usually occur in bales mainly due to better aeration, however, it would be hoped that with the careful storage of damp bales, the initial plant respiration would reduce the oxygen tension sufficiently to prevent the growth and sporulation of fungi and actinomycetes and this in turn would reduce the health hazards from the hay.

The growth of many bacterial species in anaerobic conditions is a well known fact and need not be discussed here, but more recent work has disclosed that many fungal and actinomycete species can also grow and sporulate in low oxygen tensions.

Tobak and Cooke (1968) studied the effect of various oxygen levels on the growth of 13 fungal species and found that 4% oxygen permitted normal growth, sporulation and spore germination. They also demonstrated that an accumulation of carbon dioxide was a more important factor, than low oxygen levels, in determining the effect, on fungal growth, of the atmospheric constituents and that many fungi would grow in pure nitrogen provided the vitamins biotin and thiamine were present. It was considered likely that oxygen was required for the synthesis of these vitamins.

The anaerobic growth of thermophilic fungi has been studied by various workers with some conflicting reports. Henssen (1957) reported that some fungi would grow without oxygen and that Aspergillus fumigatus grew aswell in anaerobic atmospheres as in aerobic conditions, as did Thermomyces spp, the latter fungi only doing so in the upper temperature regions of their temperature/growth curves. Kane and Mullins (1973), however recorded six species of thermophilic fungi that showed little or no growth when incubated in low oxygen concentrations. Deploey and Fergus (1975) grew Humicola grisea var thermoidea in 0.05% oxygen and found the fungi Mucor meihei and M. pusillus would grow well and sporulate in 0.3% oxygen.

Similar results have been obtained for the thermophilic actinomycetes. Henssen (1957) grew Streptomyces rectus and Thermoactinomyces thalpophilus equally well in anaerobic and aerobic conditions and she found S. thermoviolaceus grew at 60°C, only in anaerobic conditions, as did Pseudonocardia thermophila. In contrast, Deploey and Fergus (1975) tested six thermophilic actinomycetes for anaerobic growth and found no growth occurred in pure nitrogen and that growth was noticeably inhibited by oxygen concentrations of less than 1.0%, however, they did grow Thermoactinomyces vulgaris in 0.1% oxygen and found it produced

spores in an atmosphere containing 0.3% oxygen.

It appears that fungi and actinomycetes can grow and sporulate in very low oxygen tension conditions and that strictly anaerobic conditions are needed to prevent propogation. An oxygen free situation is unlikely to occur in bales of hay due to air circulation round the bales accompanied by oxygen diffusion through the hay. James et al (1928) and later Currie and Festenstein (1971) have shown that aeration greatly enhances the self-heating of damp organic material, probably due to an increase in microbial respiration, and therefore tightly packed hay bales should be less prone to heating than loosely packed bales.

e) The interaction of micro-organisms in hay

The wide range of micro-organisms which grow in moist hay have been discussed and from this a fascinating topic, which has hardly been commented on in the literature, presents itself, namely the interaction of these micro-organisms.

The most obvious interaction, and the most studied, is the competition for nutrients, which is probably an important factor in the microbial succession in hay, as has been previously discussed. Hedger and Hudson (1974) studied the effect of various thermophilic and thermotolerant fungi on the growth of the non-cellulolytic Thermomyces lanuginosa on cellophane and filter paper and noted three types of reaction. Firstly, there was no alteration of growth caused by the other fungi, which occurred with non-cellulolytic fungi. Secondly, Thermomyces lanuginosa was overgrown, by some cellulolytic fungi and thirdly other cellulolytic fungi caused rapid growth and

sporulation of Thermomyces lanuginosa. Enebo (1949 and 1951) described three thermophilic bacteria which degraded cellulose symbiotically. Only one of these bacterial species was cellulolytic, however, it had a considerably lower cellulolytic activity when grown alone than when grown with the other two non-cellulolytic species, and these, in turn, depended on the cellulolytic bacterium for their supply of carbohydrate, when only cellulose was available.

Many relationships similar to these are likely to occur in an environment with a complex microflora, not only between related micro-organisms but amongst the many different types of organism which exists there. Other polymers, besides cellulose, are also probably involved, including proteins.

The production of heat, water and carbon dioxide by micro-organisms affects the growth of the microbial flora, as does the removal of oxygen, these points already having been discussed.

Many actinomycete and fungal species are known to produce compounds, commonly known as antibiotics which inhibit the growth of other microbial species, and it would appear likely that many such compounds are produced in deteriorating hay, and they could have an effect on the development of the microbial flora.

Further interactions could be caused by the production of extra-cellular enzymes by certain micro-organisms, which could then act directly upon other members of the microflora. Possible examples could be the production of chitinases by some actinomycete spp, and chitin has been used as a selective carbon source in agar media for the isolation of actinomycetes. Price and Storck (1975) describe an enzyme produced by a streptomyces spp, which could degrade chitosan

which can account for up to 30% of the dry weight of the cell wall of certain phycomycete fungi. These extracellular enzymes could then lyse fungal cell walls and Okazaki and Iizuka (1970) showed that the living mycelium of the fungus Thermomyces lanuginosa was lysed by enzymes produced by a thermophilic actinomycete.

Finally, the addition of preservative to hay could create even further microbial interactions, because the more resistant micro-organisms would grow first utilising the readily available nutrients and possibly degrading the preservative, and this in turn, would permit the growth of the organisms less resistant to the preservative.

MATERIALS AND METHODS

All the methods described in this report were used routinely in this work.

Results which were used in the designing of these methods are described in the results section of this thesis.

SECTION A - HANDLING OF MICRO-ORGANISMS

a) Media employed

Micro-organisms isolated from hay required for further study, were grown on one of the following media. Any specialised media employed are described in the appropriate section.

Mesophilic fungi were grown on Malt Extract Agar and Oxoid Czapek Dox Agar.

Malt Extract Agar	Malt Extract (Oxoid)	20g
	Agar (Oxoid)	15g
	Distilled Water	1 litre
	p.H 5.5 (approx)	
Oxoid Czapek Dox Agar	Sodium nitrate	2.0g
	Potassium chloride	0.5g
	Magnesium glycerophosphate	0.5g
	Ferrous sulphate	0.01g
	potassium sulphate	0.35g
	Sucrose	30.0g
	Agar (Oxoid)	12.0g
	Distilled water	1 litre
	p.H 6.8.	

Thermophilic fungi were grown on Yeast Glucose Agar (Cooney and Emerson 1964).

Yeast Extract (Oxoid)	5.0g
Glucose	10.0g
Agar (Oxoid)	20.0g
Tap water	1 litre
p.H. 7.0	

Actinomycetes were grown on Oxoid Nutrient Agar and Oatmeal Agar (Schirling and Gottlieb 1966).

Nutrient Agar	Oxoid Nutrient broth	13.0g
	Agar (Oxoid)	15.0g
	Distilled Water	1 litre
	p.H adjusted to 7.5 with 1N NaoH	

Oatmeal Agar	Oatmeal	20.0g
	Agar (Oxoid)	18.0g
	Distilled water	1 litre

20.0g of oatmeal were boiled in 1 litre of distilled water for twenty minutes, then filtered through cheesecloth and distilled water was added to bring the volume back to 1 litre. 1.0 ml of the trace salts solution was added and the p.H adjusted to 7.2 using 1N NaoH. Finally the agar was added and liquefied by steaming.

Trace salts solution

$Fe\ SO_4\ 7H_2O$	0.1g
$Mn\ Cl_2\ 4H_2O$	0.1g
$ZnSO_4\ 7H_2O$	0.1g
Distilled water	100 mls

sterilised by membrane filtration.

Aerobic bacteria were grown on Oxoid nutrient agar (p.H 6.8). Lactic acid bacteria were grown on Keddie's medium. (Keddie 1951) which consisted of:-

Bacterial peptone (Oxoid)	5.0g
Yeast Extract (Oxoid)	5.0g
Lab-lemco (Oxoid)	5.0g

Glucose	5.0g
Tween 80	0.5 mls
Mn SO_4 $4H_2O$	0.1g
Tri-potassium citrate	1.0g
Agar (Oxoid)	15g
Tap water	1 litre
p.H 5.4.	

Immediately before pouring, 90 mls of the above medium were mixed with 10 mls of buffer solution of the following composition.

0.2M acetic acid (11.55 mls of glacial acetic acid in 1 litre of solution).

0.2M sodium acetate.

The sodium acetate solution was titrated against the acetic acid until the p.H was 5.4.

Many micro-organisms isolated from hay grew well on hay infusion agar, which was prepared by boiling 20g of hay in 1 litre of distilled water for 30 minutes. The solution was cooled, filtered through whatman No 1 filter paper made up to 1 litre with distilled water and 20.0g of sucrose were added along with 15.0g of agar. The agar was liquefied and the medium was dispensed and autoclaved.

All the above media were sterilised by autoclaving at 10lbs/in ^{2} for 20 minutes.

b) Incubation temperatures

Mesophilic fungi were incubated at 25°C.

Thermophilic fungi were incubated at 45°C.

Mesophilic actinomycetes and bacteria were incubated at 25°C or

37°C depending on their isolation temperature.

Thermophilic actinomycetes and bacteria were incubated at 60°C.

During the incubation of the thermophiles, the air inside the incubators was kept moist using trays of cotton wool soaked in distilled water.

c) Stock cultures

All the micro-organisms studied in this work were isolated from hay.

Stock cultures were grown on standard agar slopes using the media described. They were incubated at their isolation temperature until good growth was visible and stored at 4°C. It was found that with many of the thermophiles, sub-culturing every month was necessary to prevent loss of viability.

d) Identification of micro-organisms

i) Fungi

Fungal cultures were grown on the appropriate agar and were directly observed microscopically every two or three days using a long working distance objective. Slides for microscopic examination were prepared either by using lactophenol and cotton blue as the mounting fluid, or by gently pressing the glued surface of a small piece of sellotape on the culture and then adhering the tape to a glass microscope slide.

In cultures where spores were being produced but identification was not certain using the above techniques, then slide cultures of the fungi were prepared as follows.

A drop of sterile liquid agar medium was placed on a sterile glass microscope slide and after the agar had solidified, it was aseptically divided into halves, leaving a 2-3 mm gap. One piece of the agar was inoculated with the fungus and a sterile cover slip was placed on top of both pieces of agar. The slide was then placed on a U-shaped glass rod in a glass petri dish along with a little 15% glycerol in water solution, this apparatus being pre-sterilised by autoclaving. The apparatus shown in fig 4 was thus obtained.

The slides were incubated and examined microscopically every day for spore formation, concentrating on the gap between the agar pieces.

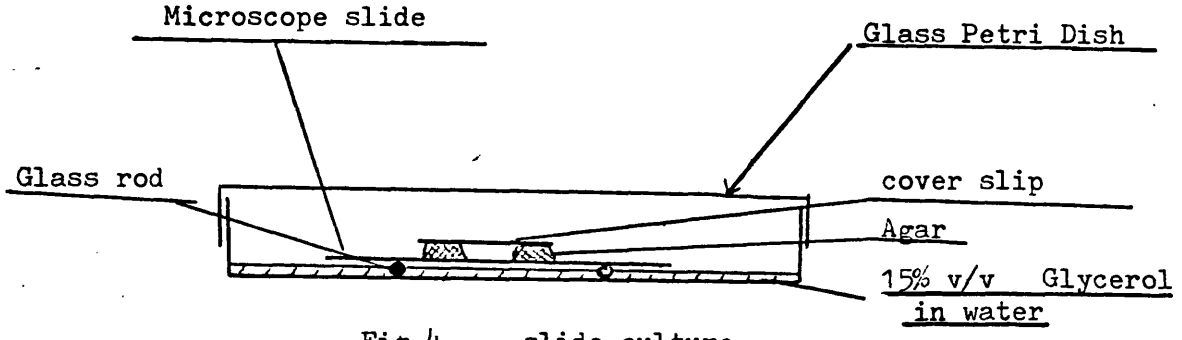


Fig 4 - slide culture apparatus

ii) Actinomycetes

Actinomycetes were inoculated by streaking onto Oxoid nutrient agar or oatmeal agar plates, which were then incubated. They were

examined microscopically every two or three days by viewing the colonies on the agar surface directly using either a long working distance objective or an oil immersion lens, these high magnifications usually being necessary for a clear picture of the spore formations.

Thermophilic actinomycetes were identified by their spore formations and colony characteristics using the works of Corbaz et al (1963) and Cross (1968) as references.

iii) Bacteria

Bacterial identification in this project was very limited, the only criteria being used were:-

a) General morphology and Gram reaction using Gram stained smears on microscope slides.

b) Motility using the hanging drop technique.

c) Catalase reaction determined by the addition of hydrogen peroxide.

d) Bacteria isolated on Keddies medium (Keddie 1951) were considered to be lactic acid bacteria.

d) Pseudomonads were differentiated from other Gram-ve rods using the arginine test of Thornley (1960) outlined below.

The medium used consisted of

Peptone	1.0g
Sodium chloride	5.0g
K_2HPO_4	0.3g
Phenol red	0.01g
Arginine Hcl	10.0g
Agar	3.0g
Distilled water	1 litre
p.H 7.0	

10 mls of the medium were dispensed in test tubes, two tubes being prepared for each bacteria under test. One of the tubes was sealed with vaseline and all the tubes were autoclaved at 10lb/ in ^{2} for 10 minutes. After cooling the tubes were stab inoculated and incubated at the isolation temperature for 10 days at 25°C, 7 days at 37°C or 3 days at 60°C. After incubation the tubes were examined and the bacteria which caused an alkaline blue colour formation in the open and sealed tubes were considered to be Pseudomonads.

SECTION B - THE HANDLING OF HAY SAMPLES

a) Microbiological counting

A major part of this work concerned the estimation of microbial numbers in hay. It was felt throughout this study that these estimations were always susceptible to large errors mainly due to the inhomogenous nature of hay.

i) Washing technique

This method was used for the estimation of total microbial numbers in hay, during the earlier part of this work, but was later discarded for actinomycete and fungal spore counts because the air sampling method, described later, was considered more suitable being less tedious and more effective at separating actinomycete spores and bacteria. This washing method was, however, retained for estimating bacterial numbers in hay throughout this work.

1) Media employed

For general aerobic bacteria estimations, nutrient agar containing 0.5 mg/ml of actidione to prevent fungal growth, was used. The actidione was dissolved in water (2.0% w/v), sterilised by membrane filtration and added to the medium immediately before pouring.

To estimate lactic acid bacteria the medium of Keddie (1951) was employed, again containing 0.5 mg/ml of actidione.

For estimating coliform bacteria, Oxoid desoxycholate agar was used, which consisted of:-

Oxoid lab-lemco powder	5.0g
peptone	5.0g
lactose	10.0g
Sodium citrate	5.0g
Sodium thiosulphate	5.0g
ferric citrate	1.0g
Sodium desoxycholate	2.5g
Neutral red	0.025g
Agar	15.0g
Distilled water	1 litre
p.H.	7.0 approx

This medium must not be autoclaved, but steamed thoroughly.

To estimate anaerobic bacterial numbers the method of Ingram and Barnes (1956) was used, the medium being Differential Reinforced Clostridial Medium (D.R.C.M), which consisted of :-

peptone	10g
lab-lemco (Oxoid)	10g
sodium acetate $3H_2O$	5g
Yeast Extract (Oxoid)	1.5g
Soluble starch	1.0g
Glucose	1.0g
L (-) cysteine	0.5g
Distilled water	1 litre

p.H 7.1 - 7.2 using 1N NaoH or 1N HCl.

The starch was added to the agar by mixing it to a paste in a little cold water, then a further 200 mls of water were added and the mixture was boiled for one minute and finally added to the rest of the medium.

Immediately before the medium was used, a sterilised mixture of 4% sodium sulphide and 7% ferric citrate was added to it at the rate of 2 ml/50 mls of medium. This mixture was sterilised by membrane filtration and was freshly prepared each week.

The anaerobic bacteria were estimated by adding 0.1 mls of the hay washings to a sterile test tube containing 10 mls of liquid D.R.C.M at 40°C. A piece of sterile black opaque tube was placed down the middle of the tube and after mixing, the medium was sealed from the air by adding a layer of sterile liquid paraffin to the surface.

After incubation, bacterial colonies could be clearly seen using a direct overhead light for illumination.

2) Method

Hay samples of known weight containing between 5g and 10g of dry hay, were placed in the blender of a Kenwood Chef food mixer, along with 200 mls of sterile distilled water. The mixer was operated at mark 3 for 1 minute and the resulting solutions were serially diluted down to a dilution of 10^{-6}, using sterile distilled water. The inside of the blender was washed with 500 mls of sterile distilled water between hay samples to remove the majority of bacteria present.

0.1 ml aliquots of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were used

for the general aerobic bacterial counts using a standard pour plate technique.

The 10^{-1} 10^{-2} and 10^{-3} dilutions were used for the lactic acid bacterial, anaerobic bacterial and coliform bacterial estimations.

The plates were prepared in sets of three replicates and were incubated at 25°C for 10 days, 37°C for 7 days or 60°C for 3 days, before counting.

ii) Air sampling technique

The washing technique was very tedious and problems were encountered when estimating actinomycete spore numbers because the plates were usually overrun by bacteria. To overcome these problems an air sampling technique was devised based on the work of Gregory and Lacey (1963a) using an Anderson Air Sampler (Anderson 1958).

1) Preparation of agar plates

Fungal spore numbers were estimated using malt extract agar containing 40 units/ml of penicillin and 80 units/ml of Streptomycin. The antibiotics were dissolved in distilled water, sterilised by membrane filtration and added to the medium immediately before pouring.

For estimating actinomycete spore numbers a half strength Oxoid nutrient agar medium containing 0.5 mg/ml of actidione was used.

To obtain the correct distance between the holes in the Anderson

air sampler plates and the agar surface exactly 28 mls of agar were dispensed into each petri dish using a Zipette (Jencons Ltd). The plates were then left overnight on the bench in order to dry and to check for contamination.

2) Method

The apparatus shown in Fig.5 was used.

The hay samples were dried, by leaving them on the laboratory bench overnight, then weighed (5g - 10g) and placed in a small wire basket. Care must be taken during this step to handle the samples as gently as possible to avoid removing spores. The wire basket was then lowered into the large plastic container, which was lined with aluminium foil to reduce the static attraction of the spores to the container wall. A small electric motor and fan were bolted to the inside of the container's lid, this motor also being covered in aluminium foil. The glass sampling tube passed through the lid and was connected to the Anderson air sampler by means of wide bore rubber tubing.

The electric motor was switched on and the wire basket, containing the hay sample, was raised and lowered rapidly for one minute, knocking it vigorously against the bottom of the container to remove as many spores from the hay as possible. The fan was then switched off and the Anderson air sampler pump was operated for five seconds in order to flush the air sampler and tubing with the spore suspension inside the container.

Three agar plates were placed in the bottom three positions of

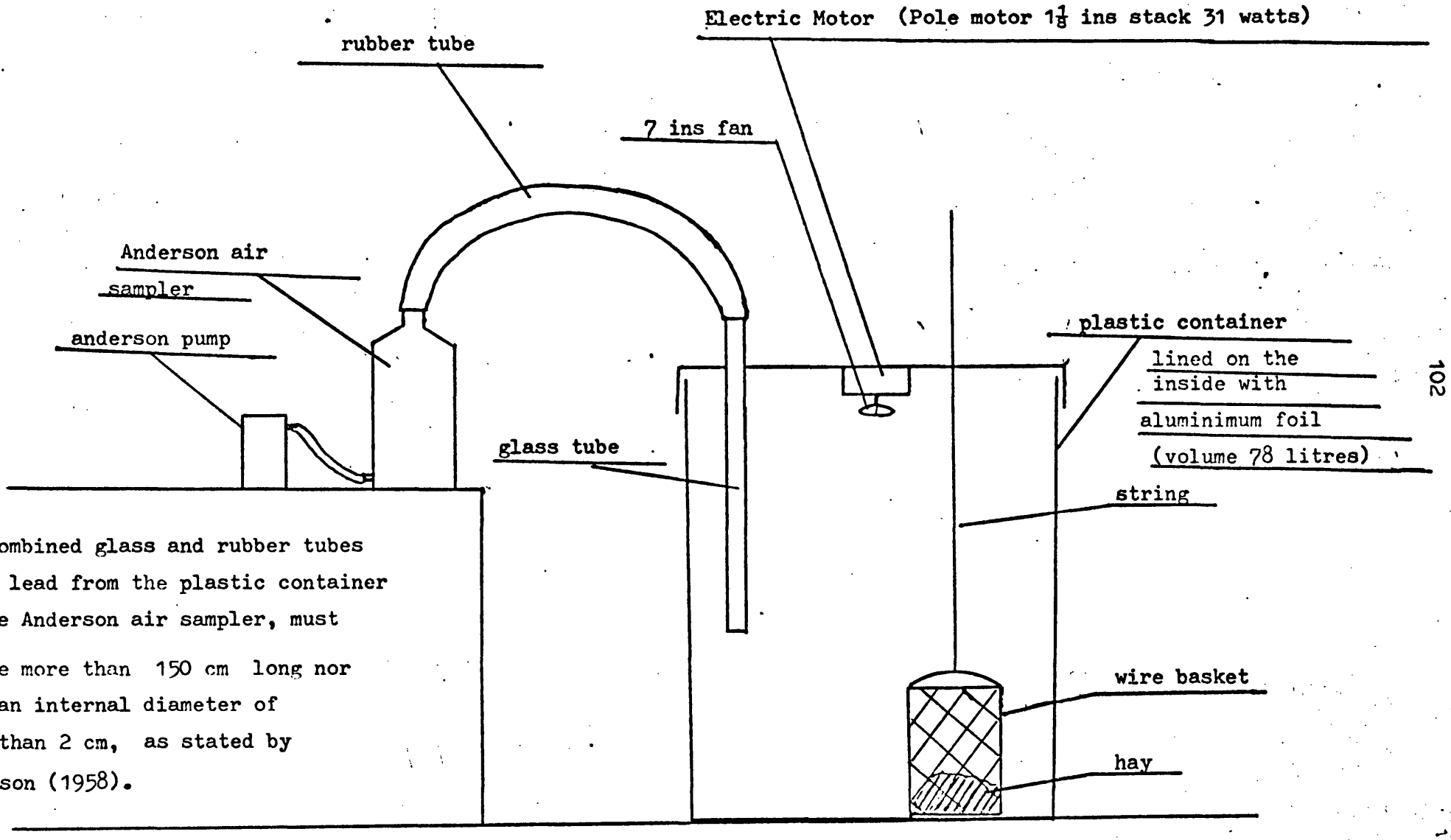


Fig 5 - Apparatus used for fungal and actinomycete spore estimates of hay

the air sampler and the pump was operated for a further 5 seconds to draw spores onto these plates. This operation was repeated until three sets of three half strength nutrient agar plates and two sets of three malt extract agar plates were inoculated from each hay sample. These plates were then incubated as follows.

The half strength nutrient agar plates	1 set at 60°C for 3 days
	1 set at 37°C for 7 days
	1 set at 25°C for 10 days
The malt extract agar plates	1 set at 45°C for 4 days
	1 set at 25°C for 10 days

After incubation the plates were counted and the number of spores per gram of dry hay calculated.

Throughout the sampling procedure an agar plate was kept in the top position of the Anderson air sampler in order to remove any incoming pieces of debris. This plate was replaced after every five hay samples due to drying.

Between each hay sample, the container and sampling tubes were thoroughly flushed out using a compressed air line, this operation being performed in the open to prevent a spore build up in the laboratory. The air sampler was flushed out by disconnecting the sampling tube and operating the air sampler pump for five seconds. When sampling was complete, the air sampler and sampling tube were placed in the container along with 25 mls of propylene oxide and the lid was placed on the container to ensure sterilisation.

The Anderson air sampler plates gave viable spore counts, but some total spore number estimates were obtained using a cascade impacter sampler which was also operated for five seconds with each

hay sample using the method of operation as described by May (1945).

b) Determination of micro-organisms in hay by chemical estimations

Counting techniques for the estimation of microbial numbers in hay were tedious and time consuming and it was felt that spore numbers were unlikely to be directly related to mycelial weight, therefore it was considered that the determination of certain chemical constituents, specific to micro-organisms, might prove to be more convenient.

The constituents chosen in this work for such determinations were chitin for fungal estimations and diaminopimelic acid for a combined bacterial and actinomycete estimation.

i) Hay hydrolysis

For chitin and diaminopimelic acid determinations the hay needs initially to be acid hydrolysed by the following method.

10g of oven dried hay were placed in a large pyrex boiling tube along with 100 mls of 6N HCl. The tubes were sealed with ground glass stoppers, which were held in position with wire, and heated at 105°C for, 6 hours with chitin estimations, 16 hours for diaminopimelic acid determinations. The samples were then filtered through Whatman No 1 paper, the excess solution being washed off the remaining solid with three 20 ml volumes of distilled water. The filtrate was finally made up to 200 mls with distilled water.

ii) Chitin estimation

The acid hydrolysis converted fungal chitin in the hay to

glucosamine which was estimated as follows.

20 mls of the filtrate were neutralised using 1N NaoH and made up to 100 mls with distilled water. The glucosamine content of the solution was then determined by one of two methods.

a) A method based on that of Morgan and Elson (1934) as modified by Levvy and McAllan (1959).

The reagents used were.

Solution A - a 1.5% (v/v) solution of acetic anhydride in acetone. This mixture was stable for only 30 minutes.

Solution B - A borate buffer prepared by adding a solution of boric acid to a 0.7M sodium borate solution until the p.H was 9.2.

Solution C - 10g of dimethyl -p- aminobenzaldehyde were dissolved in 100 mls of a 12.5% HCl solution in glacial acetic acid. One volume of this solution was diluted with nine volumes of glacial acetic acid prior to use.

0.5 mls of the test solution was placed in a clean test tube along with 0.1 mls of solution A and 0.5 mls of solution B. The tube was heated in a boiling water bath for three minutes then 6 mls of diluted solution C were added and this mixture was heated at 40°C for 20 minutes. Finally the optical density was measured at 480 mμ in a 1 cm glass cuvette with a Pye Unicam SP500 spectrophotometer. The glucosamine concentration was determined from a standard curve prepared over the range 0.0 - 2.0 mg/ml of

glucosamine hydrochloride in a 0.6M sodium chloride solution.

b) A method based on that of Tsuji et al (1969) which used five reagents.

Solution A - 5% sodium nitrite
Solution B - 5% potassium hydrogen sulphate
Solution C - 12.5% ammonium sulphamate
Solution D - 0.5% 3-methylbenzothiazolone hydrazone hydrochloride
Solution E - 0.5% ferricchloride

Reagents D and E were freshly prepared every three days and were stored in a refrigerator.

Firstly the test solutions were diluted x 20 with distilled water, and then 1.0 ml of the diluted solution was placed in a clean test tube along with 1.0 ml of solutions A and B. The mixture was left standing for 15 minutes with occasional shaking. 1.0 ml of solution C was added and the mixture shaken for five minutes. 1.0 ml of Solution D was next added and the mixture allowed to stand for one hour, after shaking, and finally 1.0 ml of solution E was added and the optical density was measured at 650 mμ after 30 minutes. A one centimeter glass cuvette was used along with a Pye Unicam SP500 spectrophotometer.

The concentration of glucosamine was determined from a standard curve prepared over a range of 0 to 30 mg/ml of glucosamine hydrochloride in a 0.03M sodium chloride solution.

iii) Diaminopimelic acid determination

This method was based on that described by Czerkawski (1974).

Four solutions were prepared.

Solution A - A citrate buffer at p.H 2.0 prepared by adjusting the p.H of a 0.1M citric acid solution with dilute HCl.

Solution B - A citrate buffer at p.H 3.4, prepared by mixing 73 mls of 0.1M citric acid solution with 27 mls of a 0.1M trisodium citrate solution.

Solution C - A citrate buffer at p.H 4.2 where 54 mls of a 0.1M citric acid solution were mixed with 46 mls of a 0.1M trisodium citrate solution.

Solution D - 2.5g of ninhydrin were dissolved in 60 mls of glacial acetic acid and 40 mls of 6M phosphoric acid.

20 mls of the filtrate were evaporated to dryness over a steam bath and the residue was dissolved in 1.0 ml of Solution A. This mixture was transferred to a small column (5 cms x 1.4 cms) of Amberlite 120 and washed in with a further 1.0 ml of Solution A. 100 mls of Solution B were passed through the column and discarded, followed by 30 mls of Solution C which were collected. 2.0 mls of the Solution C eluate were mixed with 4 mls of solution D in a clean test tube and heated in boiling water for 5 minutes. The optical density was measured at 425 mμ in 1 cm glass cuvettes with a Pye Unicam SP500 spectrophotometer.

The concentration of diaminopimelic acid was then calculated from a standard curve prepared over the range 50 - 1000 mg/ml.

After use the column was regenerated with 100 mls of 0.2N NaoH followed by 100 mls of Solution A.

c) Determination of the chitin content of fungi and the diaminopimelic acid content of bacteria and actinomycetes

The agar medium employed in these experiments was hay infusion agar.

It was decided to study the variation in the chitin content of fungi growing on the surface of the agar medium, and the variation in the diaminopimelic acid content of bacteria and actinomycetes also growing on the surface of agar. The use of submerged cultures was discarded because the chemical content of micro-organisms varies between submerged growth and surface growth (Philips 1974), and micro-organisms would grow on hay in the aerial form.

The apparatus described by Philips (1974) was used.

Cellulose nitrate filters with a 0.10μ pore size were obtained from Howe and Co Ltd, weighed and sealed onto glass rings (60 mm diam x 40 mm deep) using silicone rubber cement. These were placed in crystallising dishes and autoclaved at 10lb/in ^{2} for 10 minutes. Further crystallising dishes containing either 30 mls or 50 mls of agar medium were autoclaved at 10lbs/in ^{2} for 20 minutes and after cooling, the membranes were aseptically placed on the agar surface. The membranes were inoculated with either a loopful of fungal or actinomycete spores or a loopful of bacterial cells, these inocula having been scraped off a surface colony on a hay infusion agar plate. The inocula were then spread out as much as possible in order to obtain a lawn growth after the dishes had been incubated as follows.

Thermophilic actinomycetes and bacteria	(50 mls of agar medium)	60°C for 4 days
Mesophilic actinomycetes and bacteria	(30 mls of agar medium)	37°C for 10 days or 25°C for 14 days
Thermophilic fungi	(50 mls of agar medium)	45°C for 7 days
Mesophilic fungi	(30 mls of agar medium)	25°C for 14 days

After incubation the membranes were removed from the agar, dried to constant weight at 80°C and reweighed, thus the microbial dry weight could be determined by difference.

Finally, 1.0g of the dried microbial cells was scraped off the membrane and hydrolysed with 10 mls of 6N Hcl at 105°C for 6 hours with the fungi and for 16 hours with the bacteria and actinomycetes. The amounts of glucosamine and diaminopimelic acid present were then determined as previously described.

d) Hay moisture content determination

In order to determine the moisture content of large quantities of hay, for example a bale or the contents of the dewar flasks in experiments described later, at least three samples of 50g to 100g in weight were used, large samples being needed due to the inhomogenous nature of this material.

These samples were placed in preweighed aluminium trays, weighed, and dried in an oven at 80°C for 48 hours and then reweighed. If a drying temperature higher than 80°C was used then there arose the danger of obtaining high moisture content values due to the driving off of volatile components in the hay. At lower temperatures it would have taken too long to evaporate all the water off large moist hay samples.

Freeze drying as a means of moisture determination of hay was considered impractical due to the large sizes of the samples, and the use of moisture meters was discarded because they were found to give inaccurate results, this latter point being discussed in more detail in the results section of this work.

e) The determination of organic acids on hay

i) Volatile fatty acids

During the course of this work the levels of organic acids especially propionic acid, on hay, had to be determined in order to follow their persistance on hay and to study their distribution.

The biggest initial problem was extracting the propionic acid from the hay into a solution in water. The first method tried was to soak the hay in 0.6N H_2SO_4 for seven days, extract the propionic acid into ether and concentrate it down by evaporation of the ether, then the propionic acid was estimated using the Gas liquid chromatographic method described later. This extraction procedure was tedious and gave erratic results therefore a later method, based on that of Karasz and Hallenbeck (1972) which proved more dependable, was used.

20g of the hay were cut into approximately 20 mm lengths and placed in a Kilner jar along with a 50 ml beaker containing 10 mls of a 1.0N NaOH solution. A known amount of n-butyric acid, which was approximately the same as the propionic acid expected on the hay, was added to the hay as a standard and the jar was sealed by tightly screwing on the lid. The addition of n-butyric acid was omitted with samples having very low levels of propionic acid ($<0.05\%$) because traces of n-butyric acid occur naturally on hay. The kilner jar

was heated at 90°C, in an oven, for at least 24 hours, then cooled in air and the lid and beaker were removed. The contents of the beaker were acidified to a p.H of less than 1.0 using conc. HCl and the levels of propionic acid and n-butyric acid present were determined by the following gas liquid chromatogram method.

Gas liquid chromatogram determination of volatile fatty acids.

This method was based on that of Huysteen (1970).

A Pye Unicam Series 104 gas liquid chromatogram was used with a chromosorb 101 column of 100-120 mesh. The carrier gas was nitrogen at a flow rate of 60 mls per minute, the column temperature was 200°C and the detector temperature was 280°C.

It was found the column did not give exactly the same result each time it was used and therefore, at the beginning of each day, a standard curve was prepared by injecting, 1.0 µlt samples of a mixture of propionic acid and n-butyric acid at various known weight ratios, onto the column. The propionic acid peak heights were then plotted against the weight ratio of the acids.

After preparing the standard curve, 1.0 µlt of the unknown samples was injected into the column and from the known amount of n-butyric acid which had been originally added to the hay sample, the standard curve and the propionic acid peak height, the weight of propionic acid originally on the hay sample could be calculated.

Some difficulties were experienced with hay samples having less

than 0.02% propionic acid due to interference from the relatively large quantities of naturally occurring acetic acid on the hay, the peaks of which, tended to merge with the propionic acid peak on the traces.

ii) Sorbic acid

Sorbic acid is less volatile than propionic acid and therefore it could not be extracted from hay using the above method, and an acid soaking method had to be used.

12.5g of the hay were soaked with 100 mls of 0.6N H_2SO_4 for seven days. The mixture was filtered through Whatman No 54 filter paper and the hay washed with distilled water until the filtrate volume was 100 mls. The sorbic acid level in the filtrate was then determined by the method of Wilamowski (1971).

Three solutions were required.

Solution A	-	0.3N H_2SO_4
Solution B	-	147mg of potassium dichromate dissolved in 100 mls of distilled water.
Solution C	-	250 mg of thiobarbituric acid were dissolved in 5 mls of 0.5N NaOH solution in a 50 ml volumetric flask, by swirling under hot water. 20 mls of water and 3 mls of 1.0N HCl were then added and the volume made up to 50 mls with distilled water. This reagent was prepared fresh daily.

2.0 mls of the filtrate were placed in a clean test tube along with 1.0 mls of solution A and 1.0 mls of solution B. The mixture was heated in a boiling water bath for exactly 5 minutes, cooled in

cold water, 2.0 mls of Solution C were added and it was then reheated at 100°C for a further 10 minutes. The optical density at 532 mμ was measured using 1 cm glass cuvettes with a Pye Unicam SP500 Spectrophotometer.

Filtrates with low levels of sorbic acid (<50 p.p.m) showed some interference from hay chemicals and, therefore, after the above procedure, were scanned using a Pye Unicam SP1500 to determine the sorbic acid levels accurately. The sorbic acid having an absorbance maximum at 532 mμ .

f) Determination of the p.H and buffering capacity of hay

When hay deteriorates, the p.H changes and therefore an accurate measure of hay p.H was frequently required. The main problem encountered when measuring the p.H of hay was that only a relatively small weight of hay could be mixed with a certain volume of water.

The method adopted in this work, was to weigh out 50g of hay dried at room temperature (The hay must not be heated or its p.H value will rise) and to subdivide it into five approximately equal portions. The first portion was placed in a Kenwood Chef food mixer blender with 500 mls of distilled water, the mixer was then operated at its maximum speed for 30 seconds. The second hay portion was then added and the process repeated until all the hay had been used. The p.H of the solution was then measured using a Pye Unicam model 291 p.H meter.

To estimate its buffering capacity, 50g of room dried hay were blended, as described above, with 1 litre of distilled water. 1.0

ml of 1N H_2SO_4 or 1N propionic acid were pipetted into the mixture, thoroughly mixed in by stirring and the p.H value was measured as described previously. This process was repeated until 10.0 mls of the acid had been added. The p.H values obtained were then compared with those of 1 litre of distilled water to which 0.1N H_2SO_4 or 0.1N propionic acid had been added.

Results were obtained, for comparison, using deteriorated and fresh hays, treated with both H_2SO_4 and propionic acid, in order to determine the comparative buffering capacities of the hays, and the comparative strengths of the acids.

g) Preparation of fluorescent dye solutions and their estimation on hay

In order to assess the spray pattern obtained using hay preservative applicators, solutions of fluorochromes in water were prepared and sprayed onto hay using the same equipment as for the preservatives. Unfortunately, these solutions would probably have different physical properties to the preservatives and therefore would not give the same spray pattern, this point being discussed in more detail later in this report.

Two types of fluorochrome were employed.

a) A solution of the water soluble dye primuline (Hopkin and Williams) was prepared by dissolving 20g of the dye in 18 litres of warm tap water. 40g of polyvinyl alcohol were added to 2 litres of cool water which was heated and stirred until the chemical had dispersed. The two solutions were then mixed to give a 0.1% primuline concentration in a 0.2% polyvinyl alcohol solution.

b) Solutions of the pigment tracers (Haeffner and Co.Ltd) were prepared as described by Sharp (1974).

100g of the M.F. series of the pigment were added to 200 mls of a 0.05% (v/v) solution of Tween 80 in water. The mixture was thoroughly agitated until the pigment was completely wetted and then a few drops of silicone anti-foam (B.D.H chemicals Ltd) were added. The mixture was diluted to 20 litres with tap water to give a 0.5% pigment suspension.

To test the comparative visibilities of these solutions on hay, under ultra-violet light, firstly, single blades of grass were dipped into the solutions and secondly, the dye solutions were sprayed onto small hay samples (approximately 500g) at rates of 0.5% and 1.0%, using the apparatus shown in fig 6.

The grass blades and hay samples were then viewed under a 125 watt ultra-violet lamp (Gallenkamp Ltd) in a dark room, and visual comparisons were made, ensuring that the hay dried as little as possible before the assessments.

SECTION C - SCREENING OF POTENTIAL HAY PRESERVATIVES

Various compounds, which were considered to have potential as hay preservatives, were screened for their antimicrobial activity on hay, in three progressive stages. Firstly, using chemically defined agar media inoculated with either pure microbial cultures or mixed spore suspensions, from mouldy hay. Chemicals which were strongly antimicrobial against all hay micro-organisms, in this test, were examined for their ability to preserve small quantities of damp fresh hay stored in dewar flasks. Thirdly, a few field trials are described where bales of hay were treated with some of the more effective hay preservatives, however, the range of chemicals tested in these field trials was limited.

a) Using chemically defined agar media in plastic petri dishes

i) Media preparation

The media employed in these experiments were nutrient agar for actinomycetes and bacteria and malt extract agar for fungi. These media were prepared in buffer solutions at p.H values of 5,6,7 and 8, most hays having a p.H value within this range.

The buffers used had the following compositions.

Two solutions were prepared.

Solution A - 0.2M solution of monobasic sodium phosphate ($31.2g\ NaH_2PO_4 2H_2O$ in 1 litre of solution)

Solution B - 0.2M solution of dibasic sodium phosphate ($28.39g$ of Na_2HPO_4 or $71.7g$ of $Na_2HPO_4\ 12H_2O$ in 1 litre of solution.)

To prepare 1 litre of buffer

p.H 6.0 — Mix 438.5 mls of solution A with 61.5 mls of Solution B and 500 mls of distilled water.

p.H 7.0 — Mix 195.0 mls of solution A with 305.0 mls of Solution B and 500 mls of distilled water.

p.H 8.0 — Mix 26.5 mls of solution A with 473.5 mls of Solution B and 500 mls of distilled water.

p.H 5.0 buffer

Two solutions were prepared.

Solution A - 0.1M citric acid (19.21g in 1 litre of solution).

Solution B - 0.2M dibasic sodium phosphate (see above) Mix 243 mls of Solution A with 257 mls of Solution B and 500 mls of distilled water.

ii) Preparation of plates

The compounds to be tested were sterilised by either autoclaving at 10lb/in ^{2} for 10 minutes where suitable, for example with the volatile fatty acids, or by dissolving them in either water or ethanol, and then membrane filtering these solutions. The buffered media were prepared and sterilised by autoclaving at 15lbs/in ^{2} for 15 minutes and when they had cooled to 50^{o}C the preservatives were added to give final concentrations of 5.0%, 1.0%,0.1%, 0.01%, 0.001% and 0.0001%.

Cochrane (1958) noted that the depth of agar media in plates could affect the growth of fungal colonies and therefore in all these tests either 25 mls of medium in a normal petri dish or 8 mls in each

compartment of a three-sectioned petri dish, were dispensed using a zipette (Jencons Ltd). Where three-sectioned petri dishes were employed, different preservative levels were used in each compartment.

After inoculation and incubation the lowest preservative level which totally inhibited microbial growth was noted and a fresh series of plates were prepared using concentrations of preservative between the noted level and one tenth of the noted level, for example, if in the first stage of the experiment, 1.0% of the preservative was the lowest totally inhibitory level then in the second experiment, a range of concentrations between 1.0% and 0.1% would be prepared i.e. 0.2% 0.4% 0.6% and 0.8%.

Antibiotics or actidione were added to the media in three-sectioned petri dishes because a mixed inoculum was used.

iii) Inoculation

In experiments where pure microbial cultures were used, the micro-organisms used for inoculation were grown on either nutrient agar or malt extract agar plates. Only micro-organisms isolated from hay were used and they were grown at their isolation temperature.

Fungi were inoculated onto the plates by aseptically removing a disc, from the edge of an actively growing colony using a small cork borer, and placing it, mycelium downwards, on the centre of the agar plate. Actinomycetes and bacteria were inoculated using a standard streaking procedure with a loopful of the micro-organism scraped from an actively growing surface colony.

The use of a whole range of micro-organisms was found to make the screening procedure very time consuming, demanding on petri dishes and did not take account of preservative resistant micro-organisms, therefore it was decided to simplify the method using three-sectioned petri dishes.

These were prepared as described and then one was placed in the bottom section of an Anderson air sampler. Spores from mouldy hay were deposited on the surface of this plates using the air sampling method previously described, operating the air sampler pump for 5 seconds for each plate. This process was repeated until all the plates had been inoculated. Samples of deteriorated hay were regularly agitated in the container to ensure each agar plate received a heavy inoculum.

iv) Incubation

The plates were incubated as follows:-

Nutrient agar plates at 60°C for 4 days
or37°C for 10 days
or25°C for 14 days

Malt extract agar plates at 45°C for 7 days
or 25°C for 14 days

b) Using hay contained in 4.5 litre dewar flasks

It was hoped that hay packed into a dewar flask would undergo basically the same microbial and biochemical changes as similar hay in a bale. It was, however, known that there were many important differences including hay compaction, heat insulation and water vapour

and gas diffusion and because of these differences dewar flask experiments were used only to compare the anti-microbial activities of preservatives on hay and not to determine their effective levels at preventing baled hay from moulding.

i) Method

Fresh hay, which had not self-heated or moulded, was obtained and its moisture content was determined by oven drying as previously described. Hay which contained less than 20% moisture was considered ready for use, whereas wetter hay was spread out in a greenhouse or barn until its moisture content had fallen below 20%.

The amounts of water and preservative necessary to bring the moisture and preservative levels on the hay to the required levels, were calculated and measured out. The apparatus shown in fig 6 was then set up.

500g of the dry hay were placed in the wooden tray and were spread out as evenly as possible. The water and preservative were mixed thoroughly and placed in a 250 ml Buchner flask which was connected, via its sidearm, to a compressed air line adjusted to give a small air pressure. A rubber stopper, with a piece of flexible tubing through the middle, was pushed into the neck of the flask so that the end of the tubing was situated at the lowest point inside the flask. A nozzle size 6, marketed by Feed Service Ltd, for application of preservatives to hay being baled, was attached to the other end of the tubing.

When clip A was opened, the air pressure inside the Buchner

flask increased forcing the mixture to spray out through the nozzle. The nozzle was held by hand, and moved rapidly over the hay giving as even a coverage as possible. After approximately half the liquid had been sprayed on, the hay was turned over and the remaining solution sprayed on. The hay was then thoroughly mixed by hand and as much as possible was packed firmly around a thermometer in a clean dewar flask. When full, the dewar flasks were placed on the floor, where they would receive as little draught as possible, in a room whose air temperature was thermostatically controlled at 17°C. A further thermometer was placed close to the dewar flasks to keep a record of the ambient air temperature.

Chemicals, which were insoluble in water, including sorbic acid, paraformaldehyde and hydroxybenzoates were applied as a fine suspension in the water, the Buchner flask being continously shaken during the application.

Three samples each containing approximately 50g of dry hay were removed from the treated hay immediately before packing, weighed and then oven dried to check the final moisture content of the hay in each dewar flask. Three samples of similar size were taken from organic acid treated hay, for preservative level determinations. Samples were also taken from the untreated control hay after the water had been added, in order to determine the initial microbial numbers.

During storage the temperatures of the flasks were recorded daily, and when heating had finished in all the flasks, they were carefully emptied and the hay examined visually.

From the contents of each flask, three samples were taken for

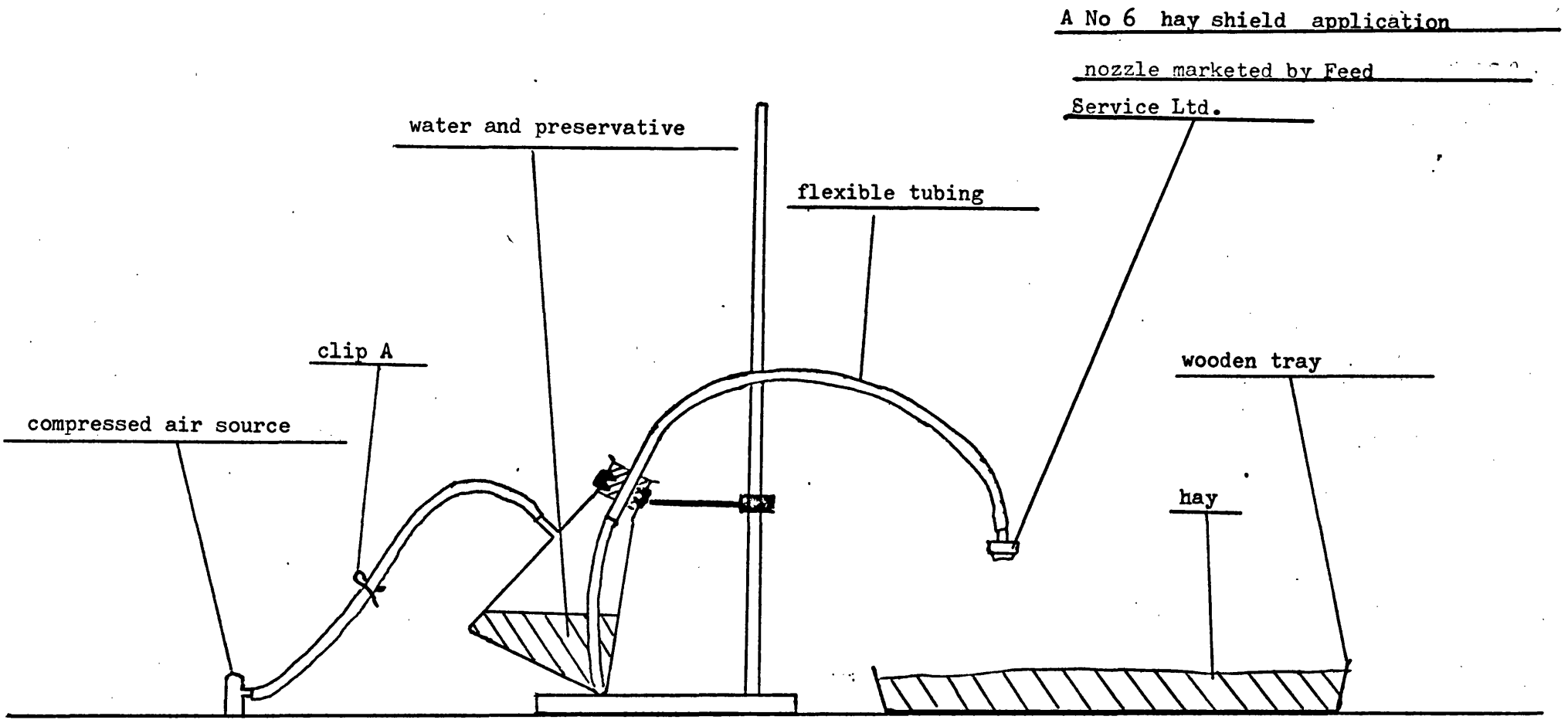


Fig 6 - Apparatus for applying water and preservatives to hay to be stored in dewar flasks.

final moisture level determinations, three more for microbial assessments and where organic acid preservatives had been used, a further three samples were taken for determination of their final levels. After the hay used for final moisture level determinations, had been dried and reweighed, 10g samples were hydrolysed with 6N HCl in order to calculate their chitin and diaminopimelic acid contents, using the methods previously described.

ii) Dewar flask experiment to study the moulding of organic acid treated hay.

One particular dewar flask experiment will be described seperately because it was designed to study the changes which took place when moist hay, treated with propionic acid and sorbic acid, moulded inside dewar flasks.

Ten dewar flasks were filled, using hay with an initial moisture content of 45% and treated with 0.9% propionic acid and 0.1% sorbic acid, as previously described.

At intervals of four days the carbon dioxide content of the gas in a flask was measured, by removing the thermometer and placing a high range carbon dioxide tube connected to a kitagawa precision gas detector (Pitman D.A. Ltd), down the hole, and then drawing 100 mls of gas through the carbon-dioxide tube.

The hay from the dewar flask was then examined as described above and in addition, a few further measurements were made.

Firstly, the number of cellulolytic micro-organisms were counted using the washing technique for bacteria and the air sampling procedure

for actinomycete spores and fungal spores. Secondly, the number of fungal and actinomycete spores which could germinate in anaerobic conditions were counted by placing air sampling agar plates in anaerobic jars filled with a hydrogen, carbon dioxide mixture free of oxygen. Thirdly, the developement of propionic acid resistant micro-organisms was followed by adding a drop of the undiluted washings, used for estimating bacterial numbers, in each compartment of a 3-sectioned petri dish and then filling the compartments with either malt extract agar or nutrient agar both buffered at p.H 6.0 and containing propionic acid levels of 0.05%, 0.1%, 0.2%, 0.4%, 0.6% or 0.8%. After incubation, the minimum totally inhibitory levels of propionic acid were noted.

Agar media employed

1) Cellulose media

Fungi (Eggins and Pugh 1962)

Ammonium sulphate	0.5g
L-asparagine	0.5g
potassium dihydrogen phosphate	1.0g
potassium chloride	0.5g
$MgSO_4$ $7H_2O$	0.2g
Ca Cl_2	0.1g
Yeast Extract (Oxoid)*	0.5g
Ball milled cellulose	10.0g
Agar (Oxoid)	20.0g
Distilled water	1 litre
p.H	6.2

* Whatman's cellulose powder was ball milled as a 4% suspension in water for 72 hours.

Actinomycetes and Bacteria (Fergus 1969)

K_2 HPO_4	1.0g
$MgSO_4$ $7H_2O$	0.3g
Bacto-peptone (Oxoid)	1.0g
Yeast extract	0.1g
Balled Milled Cellulose*	10.0g
Agar (Oxoid)	20.0g
Tap water	1 litre

p.H 7.5 with 1N KOH for actinomycetes

p.H 7.0 with 1N KOH for bacteria

2) Media for anaerobic growth (Deploey and Fergus 1975)

Fungi

glucose	10.0g	
$KH_2 PO_4$	1.0g	
$MgSO_4 7H_2O$	0.5g	
microelement solution	2.0 ml	
Yeast Extract (Oxoid)	2.0g	
Agar (Oxoid)	20.0g	
Distilled water	1 litre	p.H 6.0

Microelement Solution

$Fe (NO_3)_3 9H_2O$	723 mg
$Zn SO_4 7H_2O$	440 mg
$Mn SO_4$	203 mg
cleared with conc. H_2SO_4	
distilled water	1 litre

Actinomycetes

Bacto-peptone (Oxoid)	5.0g	
Yeast Extract (Oxoid)	3.0g	
Sucrose	5.0g	
$MgSO_4 7H_2O$	0.5g	
$Fe SO_4$	10 mg	
microelement solution	1 ml	
Hay extract	5 ml	
Distilled water	1 litre	p.H 7.0

Microelement Solution (1.0 ml)

1 mg of Fe as $Fe (NH_4)_2 (SO_4)_3$
1 mg of Zn as $ZnSO_4$
0.05mg of Mn as $MnSO_4$
0.08 mg Cu as $CuSO_4$
0.1 mg Co as $CoSO_4$
0.1 mg B as H_3BO_3

Hay extract

Boil 5g of fresh hay with 100 mls of distilled water for 20 minutes, filter through paper and make the volume up to 100 mls with distilled water.

All these media were sterilised by autoclaving at 10lbs/in ^{2} for 10 minutes.

Incubation

These sets of agar plates were all incubated as follows.

Actinomycetes and bacteria	at 60°C	for 4 days
	or 37°C	for 10 days
	or 25°C	for 14 days
Fungi	at 45°C	for 7 days
	or 25°C	for 14 days

C) Field Trials

A few of the more promising hay preservatives were tested for their ability to prevent baled hay from moulding. The experimental procedure for these trials varied in minor details, but the basic method used was similar to them all and is outlined below.

A field of grass was set aside and in suitable early summer weather it was cut and turned regularly until it was thought to be approximately at the required moisture level. The determination of the moisture content of the hay in the field was one of the main problems encountered during these trials. Oven drying could not be used because it took too long, drying of samples under infra red lamps also proved too time consuming and moisture meters were

inaccurate except for an experimental one produced by R.D.S Ltd, which estimated the moisture content of hay by measuring its electrical conductance and capacitance. The moisture levels of various hay samples were estimated by oven drying and by the R.D.S. meter and the results are discussed later in this work.

In all these field trials, except for the first one, a moisture content of 30% to 35% was aimed for, but due to many setbacks including, undependable weather, inavailability of equipment and technical faults, the actual baling moisture levels were usually not ideal.

After the equipment was operative, the baling rate and bale weights were determined and the preservative application rates calculated. The application rates of the preservatives were controlled by altering the jet sizes and pump pressure in the first two trials, but by the use of a flow meter incorporated into the pump for the remaining field trials. The chemicals were applied through one to four jets mounted above the baler pick up so that the hay was sprayed as it entered the ram chamber. An electric pump mounted in the tractor driver's cabin supplied the pressure except in the second and third trials where a carbon dioxide cylinder was used. The preservatives were transported in five gallon plastic pressure containers which were held either on the baler or in a small cradle at the front of the tractor.

When baling started, the untreated controls were first prepared, application of the first treatment was started, the next four bales were discarded and the required number of treated bales were then prepared. The treatment was then altered, the next four bales were discarded and the next set of treated bales were collected. This procedure was continued until the trial was completed.

The next stage was to apply fluorescent dye solutions to a few bales, using the same equipment as for the preservatives. The bales were then taken back to a dark room where they were examined under an ultra-violet lamp to visually assess the spray pattern.

After treatment, the bales were stacked on either polythene sheets or a bed of straw, in separate compact piles, in an open barn, the total number of bales depending on how much grass was originally available. Soil thermometers were inserted completely into the central bale of each pile for temperature recording, except in the last field trial when a Honeywell thermocouple temperature recorder was used.

Five samples were taken, from an untreated hay bale and from a final treatment bale, immediately after baling, to determine the initial moisture levels at the beginning and at the end of the field trials. Five samples were also removed from an untreated bale for initial microbial spore counts, and preservative level determinations were made on ten samples taken from treated bales. Fungal and actinomycete spore counts were estimated using the air sampling technique previously described, however, bacterial numbers were not estimated because they were thought to be less important, especially when considering the health problems of deteriorated hay.

During the heating phase of the hay baled in the first two field trials, five samples for microbial spore counts were taken from single bales, at regular time intervals, by prising the bales open and pulling the hay samples out, by hand, from within the bales, using sterile disposeable gloves. This method was later discarded because it was considered to be inaccurate due to many spores being lost from the hay samples as they were withdrawn from the bales. In the later field trials, bales were carefully broken open, when

all heating had finished, and five samples were gently removed from each bale and carefully placed in sterile plastic bags which were then taken back to the laboratory for microbial analysis. This sampling procedure was repeated after a longer storage period of three to four months.

Samples for estimating preservative levels were removed by the same methodology. The number of samples used and the length of time in storage before they were removed are detailed in the results section of this report.

SECTION D - STATISTICAL ANALYSIS

Hay is a very inhomogenous commodity, and because of this, representative sampling presents many problems. When the microbial counts of hays treated with various preservative were compared, in order to determine whether one treatment had controlled microbial growth more effectively than another treatment, a statistical analysis of the results was needed to distinguish between the differences due to treatments and the differences which occur within a treatment. All microbial counts of hay in this work, whether from field trials or dewar flasks, were statistically analysed by the technique known as analysis of variance, ($P < .05$).

a) Analysis of variance

For each set of microbial counts a table was prepared as shown in table 5.

Treatment	Counts /g dry hay (Replicates)					Total T	Replicates n	Mean T/n	T^2/n
Untreated	10	11	12	13	14	60	5	12	720
Treatment 1	1	2	3	4	5	15	5	3	45
Treatment 2	6	7	8	9	10	40	5		
Treatment 3	10	9	8	7	6		5		
Treatment 4	5	4	3	2	1		5		
						$\sum T$	$\sum n$		$\sum T^2/n$

TABLE 5

Correction Factor (C.F.) = $\frac{(\sum T)^2}{n}$

Sum of the squares (U.S.S.) = Total of all the squared counts
i.e. $(10^2 + 11^2 + 12^2 \ldots\ldots + 1^2)$

Source	degrees of freedom			
Treatments	4	$\sum T^2/n$ - C.F. = A	$A/df = A/4 = C$	C/D
Residual	20	U.S.S. - $\sum T^2/n$ = B	$B/df = B/20 = D$	X

degrees of freedom =

Treatments = Number of treatments - 1 ie 5-1 = 4

Residual = total number of degrees of freedom ie (5-1) + (5-1) + (5-1) + (5-1) = 20

X is determined from probability tables with a 0.05% probability and the treatment and residual degrees of freedom.

If $^{C}/D$ is larger than X there is a statistical difference between the treatments within the experiment. If $^{C}/D$ is less than X there are no significant differences and the means test is not used.

Means Test

To calculate whether two particular treatments are significantly different the means Test was used.

$$\text{Standard Deviation (S.D)} = Y \sqrt{(^{1}/n^{1} + {}^{1}/n^{2})D}$$

Y is determined from T tables using a 0.05% probability and the residual degrees of freedom.

n^1 = Number of replicates in one treatment.

n^2 = Number of replicates in the other treatment.

If the difference between the means $(^{T}/n)$ of the two treatments is larger than the S.D. then the two treatments were considered significantly different.

B) Coefficient of Variation

The distribution of acid preservatives in a bale of hay, was calculated by estimating the acid levels on several samples taken

from within that bale. To compare the distribution obtained using different applicators or different application rates, the coefficient of variation was calculated for these acid levels from each bale.

There are seven stages in this calculation.

1) Calculate the mean of the levels (M)

2) Subtract each value from the mean and ignore negative signs.

3) Square these differences

4) Total the squares

5) Divide the answer by the number of samples = sample variance = S.V.

6) $\sqrt{\text{S.V.}}$ = Standard Deviation = S.D.

If the two treatments being compared were different application levels, then a comparison of the standard Deviations would not give a true comparison of distribution due to the different sample sizes, therefore the Coefficient of Variation (C.V) (Karl Pearson's) needs to be calculated as follows.

7) $$\text{C.V} = \frac{100 \times \text{S.D.}}{\text{M}}$$

A treatment with a lower C.V. than another yielded a more even distribution of acid within the samples measured.

RESULTS

Actinomycetes and bacteria referred to as thermophiles were those which grew at 60°C and mesophiles were those which grow at 37°C and 25°C. Fungi that were incubated at 45°C are referred to as thermophiles in this work although they consisted of both Thermophilic and thermotolerant species as defined by Cooney and Emerson (1964).

SECTION A - THE HANDLING OF HAY SAMPLES

a) Microbiological Counting

Throughout this work an estimate of the extent to which hay had deteriorated was required, and the most frequently used method was to determine the viable microbial numbers present in the hay, which was expressed as the microbial count and consisted of the total viable fungal spore count, the total viable actinomycete spore count and the total bacterial count per gram of dry hay.

Two methods for estimating the microbial counts of hay were employed in this work, namely a washing, serial dilution and pour plate method and an air sampling technique. The comparative merits of these two methods will be discussed.

i) Washing technique

The hay samples were homogenised with sterile distilled water, then the resulting solution was serially diluted the microbial numbers were estimated by a pour plate technique. It was observed that a considerable number of the spores floated on the surface of the water

and in an attempt to overcome this problem, 0.1% of the wetting agents Teepol or Tween 80 was incorporated into the sterile distilled water. The results in Table 6 show that the wetting agents had no significant effect on the microbial counts, despite the fact that they visually appeared to wet the spores and allowed a more even suspension of spores, to be formed.

Number of micro-organisms /g of dry hay X 10^6									Incubation Temperature
Distilled water			Distilled water+ 0.1% Teepol			Distilled water+ 0.1% Tween 80			
Fungi	Actino	Bact	Fungi	Actino	Bact	Fungi	Actino	Bact	
3	70	1000	6	100	1050	6	60	900	45°C
150	400	700	500	200	700	300	400	800	25°C

TABLE 6 - The effect of Teepol and Tween 80 on estimating the microbial numbers in hay.

An additional problem encountered when using the washing technique, especially with cleaner hays, was that a large number of the nutrient agar plates were overrun by bacteria, which made accurate actinomycete spore estimation impossible. At first, an attempt to overcome this problem was made by using agar media selective for actinomycetes. Three media were tested, firstly that of Fergus (1964) which incorporated 800µg/1t of aureomycin, Secondly that of Kuster and Williams (1964) which used glycerol as the main carbon source and thirdly, one based on that of Crook et al (1950) which consisted of nutrient agar containing 0.4% sodium propionate. It was observed that the aureomycin did not reduce the number of bacterial colonies in proportion to the number of actinomycete colonies, whereas the second and third media reduced the bacterial colony numbers and not the number of actinomycete colonies,

unfortunately the reduction was rarely sufficient to permit accurate actinomycete colony counting.

ii) Air sampling technique

In an attempt to overcome these problems, an air sampling technique was developed, based on that used by Gregory and Lacey (1963a), which has previously been described in the methods and materials section.

When deteriorated hay was being handled , it was observed that dry samples appeared dusty, whereas damp samples, although appearing mouldy, did not readily give off large numbers of spores when agitated. It was therefore considered, that the air sampling method would be more accurate if the hay was dry before being sampled.

To confirm this theory, the effect of various lengths of drying time of hay samples on a bench in a room with an air temperature of 20°C was observed, on the estimated microbial counts of the hay samples.

The samples, each weighing approximately 10g, were all taken from the same bale of deteriorated hay, and were then spread out evenly over an area of approximately 1 square foot to facilitate drying. The results in fig 7 show that as the hay dried, its estimated fungal and actinomycete spore content increased and that a drying period of approximately twelve hours was required to obtain the maximum possible release of spores. The spores released being expressed as a percentage of the maximum number of spores that were counted. Therefore throughout this work when the spore counts of hay samples were determined by the air sampling technique, the hay was firstly dried overnight on the

laboratory bench.

To determine the optimum hay sample size to be employed for this method, a large hay sample (100g) was taken from a bale and dried overnight on the laboratory bench. Samples of various known weights were then taken, their microbial spore counts were estimated and the results (fig 8) show that hay samples weighing between 4g and 10g gave the maximum spore counts /g of dry hay. Small samples were considered to be less suitable because they would give less representative results and large samples (more than 10g) could not be as efficiently agitated and therefore a smaller proportion of the total number of spores present was removed by the agitation in the container. Therefore when the spore counts of hay were estimated using the air sampling technique, samples weighing approximately 10g were employed.

The results in fig 9a suggest that the spore concentration inside the plastic container increased during the first minute of hay agitation, but that after the minute, it gradually decreased presumably because the number of spores removed from the hay by continued agitation, was less than the spore loss from the container due to possibly either leakage or electrostatic attraction to the container walls. The latter explanation was considered more probable because the addition of double sided adhesive tape to the rim of the container, which sealed the gap between the lid and container, did not decrease the apparent spore loss. The settling out of the spores was not responsible for this apparent spore loss because the fan was left running throughout this experiment.

In a later experiment it was observed that the spore loss was more rapid with the fan in operation than when it was not, (fig 9b) possibly because the increased air movement aided the contact of spores with the

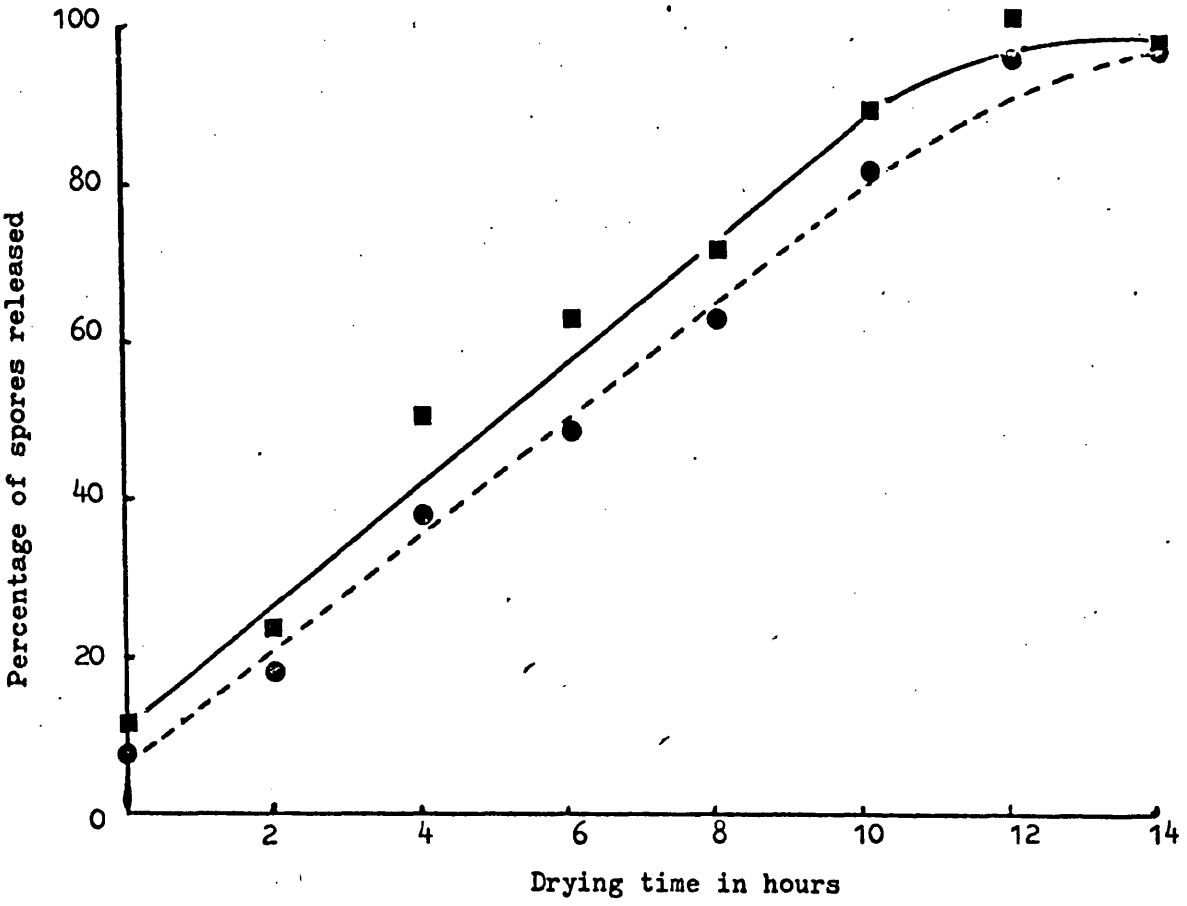


Fig 7 The effect of the drying time of hay samples at room temperature on the release of spores (■) Actinomycetes (●) Fungi

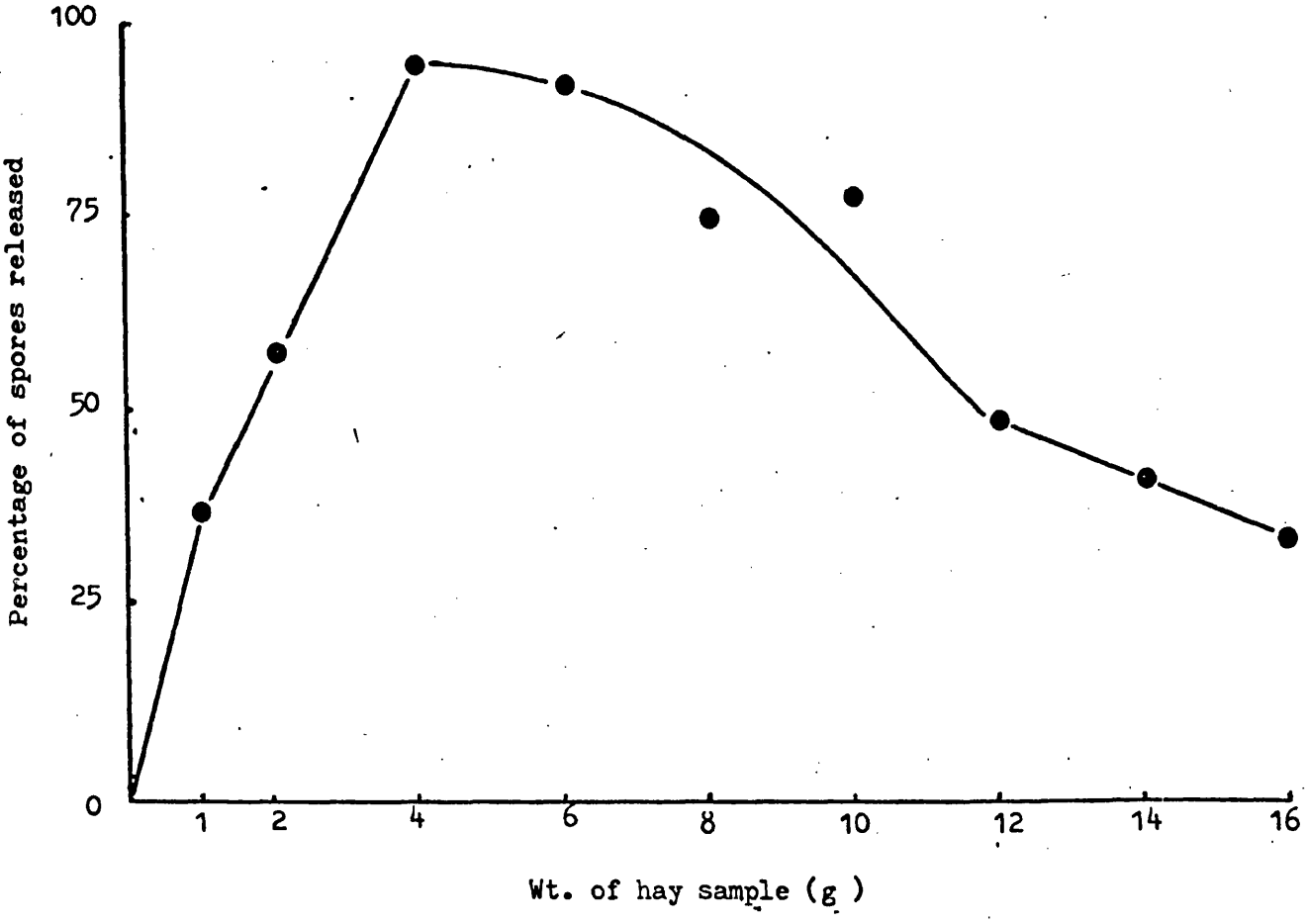


Fig 8 The effect of varying the hay sample size on the estimated microbial spore count /g dry hay.

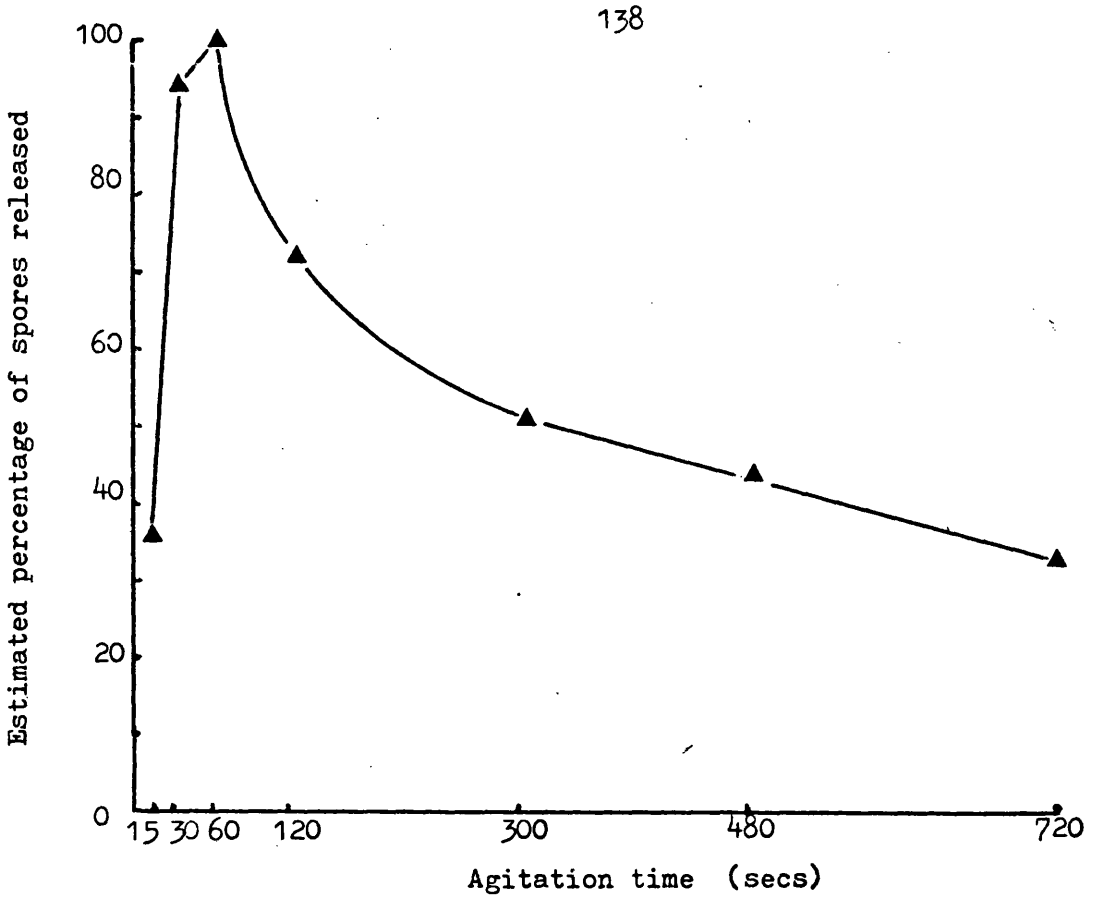


Fig 9a The effect of agitation time on the estimated microbial spore count of hay.

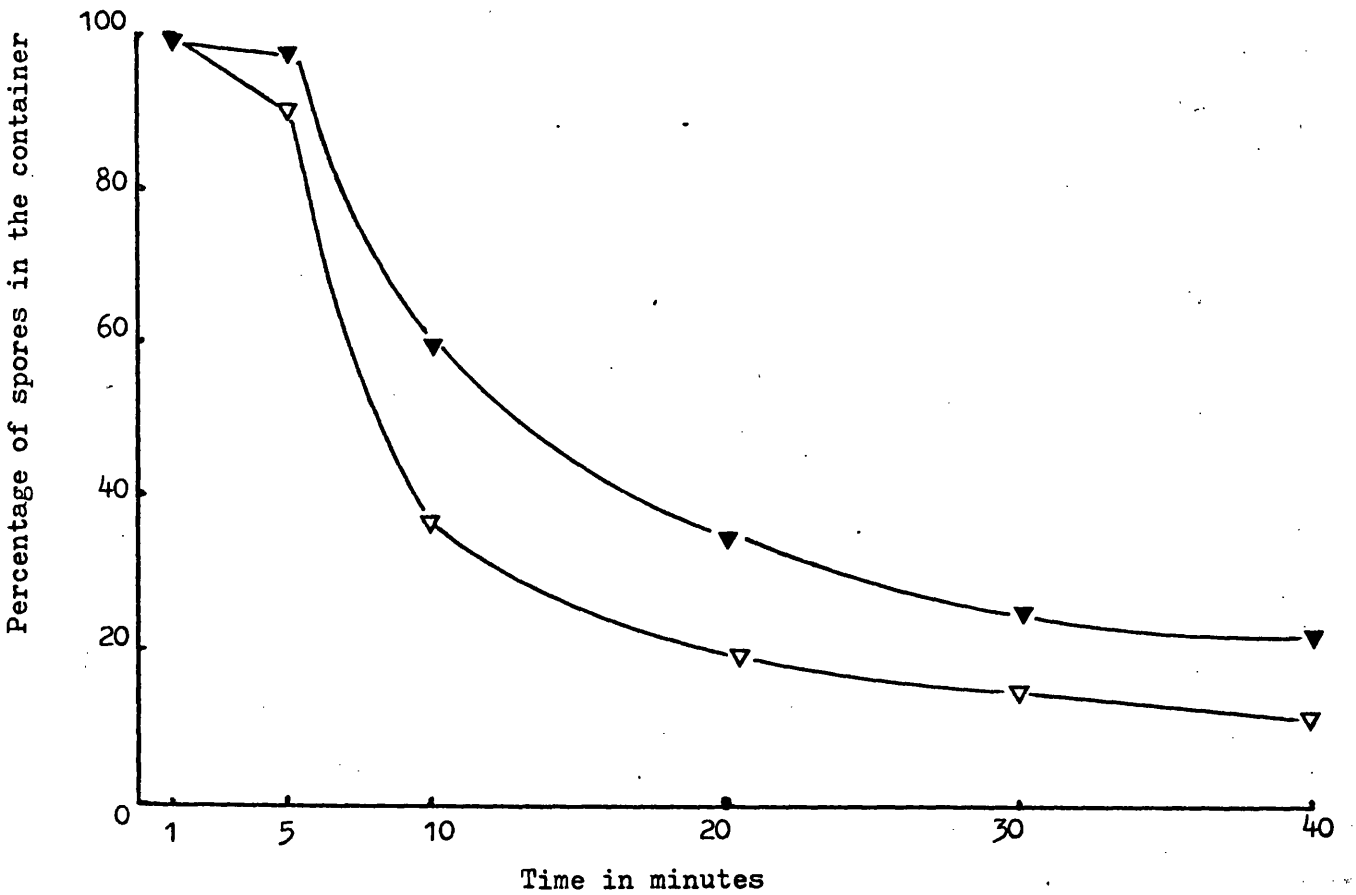


Fig 9b Spore loss from the air sampling container
(▽) Fan running (▼) Fan not running

sides of the container thus aiding electrostatic attraction.

When the spore counts of several hay samples were being determined, between each sample it was necessary to remove all spores from the apparatus. Unfortunately, this could not be done by the use of gaseous sterilants, including popylene oxide and formaldehyde, because of the time factor involved and the danger of killing spores from the following hay samples, therefore it was decided to flush all the spores out of the apparatus using compressed air. This flushing procedure was done outdoors, in order to prevent the build up of spores in the laboratory.

In order to test the effectiveness of the flushing in the removal of the spores from the apparatus, deteriorated hay samples were agitated inside the container and the spore concentration was estimated as previously described. The container and sampling tube were then cleaned with compressed air for one minute and the Anderson air sampler was flushed by operating its pump for ten seconds, with the sampling tube disconnected. The spore concentration inside the container was re-sampled and the process was then repeated. The results in table 7a suggest the cleaning procedure removed more than 99% of the spores from the apparatus, and the results in Table 7b suggest a 10 second flushing of the Anderson air sampler removed 99% of the spores from within the sampler.

	Estimated spore count /g dry hay
Before flushing	407.0×10^4
After 1st flushing	3.8×10^4
After 2nd flushing	2.1×10^4

Table 7a - Removal of spores from the air sampling apparatus by flushing with compressed air.

Time of pump operation	Estimated spore count /g of dry hay
0 secs	660×10^4
5 secs	41×10^4
10 secs	3×10^4
15 secs	2×10^4
20 secs	3×10^4

Table 7b - Removal of spores from the Anderson air sampler by operation of the pump.

To obtain a comparison between different methods of estimating microbial counts, hay samples were taken from a bale and the spore counts were estimated using the air sampling technique with firstly an Anderson air sampler and secondly a Cascade Impactor air sampler. These results were then compared with thoseobtained by the washing method and are shown in Table 8.

Sampling method	Microbial spore count /g of dry hay			
	Actinomycetes at 60°C	Actinomycetes at 37°C	Fungi at 45°C	Fungi at 25°C
Washing	24×10^6	54×10^6	80×10^6	180×10^6
Anderson sampler	18×10^4	84×10^4	28×10^4	75×10^4
Cascade impactor	Total count of 88×10^6			

Table 8 - Comparison of the estimated microbial spore content of hay by the washing technique and the air sampling technique using the Anderson air sampler and the Cascade impactor air sampler.

The washing method gave the highest, and probably the most accurate estimated spore counts probably because blending with water removed the spores from the hay more effectively than the agitation technique

employed with air sampling. However, the air sampling results did give a better guide as to the number of spores which would have been removed from the hay if it had been handled on a farm, and it is these spores which would have then been potentially hazardous to man and animals. The results obtained using the cascade impactor were considerably higher than with the Anderson sampler, and because the former air sampler estimated the total spore concentration, whereas the Anderson air sampler estimated the viable spore numbers, it would seem that the majority of the spores which were present on the hay were non-viable. These results are in agreement with those of Gregory and Lacey (1963a).

Many of the spores found, in deteriorated hay are potentially hazardous to man and in order to check whether the air sampling technique raised the spore concentration inside the laboratory to a dangerous level, the spore count of the laboratory air was estimated using an Anderson air sampler before and after the spore determinations, of a dozen deteriorated hay samples, were made. The results (Table 9) show that, although the air spore concentration did increase during sampling, it remained low. Nevertheless, as a safety precaution, suitable respirometers were worn during sampling.

	Spores / 100 litres of laboratory air				
	Actinomycetes at 60°C	Actinomycetes at 37°C	Actinomycetes at 25°C	Fungi at 45°C	Fungi at 25°C
Before sampling	0	16	57	0	12
After sampling	0	201	521	1	220

Table 9 - Spore concentration of the laboratory air, before and after sampling deteriorated hay.

b) Chemical determination of the microbial population of hay

It was hoped that the estimation of chitin and diaminopimelic acid (D.A.P.A.) in hay would prove to be a rapid, accurate and convenient means of determining the microbial content of hay, especially as the sampling procedures described previously were demanding in both time and materials.

i) Chitin estimation

The determination of chitin in casava cheese by acid hydrolysis to glucosamine and colourimetric determination of the glucosamine, has been described by Philips (1974), and the same basic method was employed to estimate the chitin content of hay.

Early in this work the glucosamine was determined by the method of Morgan and Elson (1934) as modified by Levvy and McAllan (1959). Later, this colourimetric method was compared with that of Tsuji, Kinoshita and Hoshino (1969) for interference from other hay components and the results (fig 10a & 10b) show that the latter method was more sensitive and suffered from less interference. The Morgan - Elson method gave low estimates for known weights glucosamine which had been added to hay hydrolysates, probable due to interference from amino sugar derivatives present, especially galactosamine. The method of Tsuji et al (1969) also suffered from interference probably from neutral sugars, amino acids and aldehydes but the results (fig 10b) suggest this interference was minimal partly due to the greater dilutions of the hay hydrolysates, which were employed. Philips (1974) removed the compounds which interfered with the Morgan - Elson method using an Amberlite CG120 column, however, this procedure was discarded in this

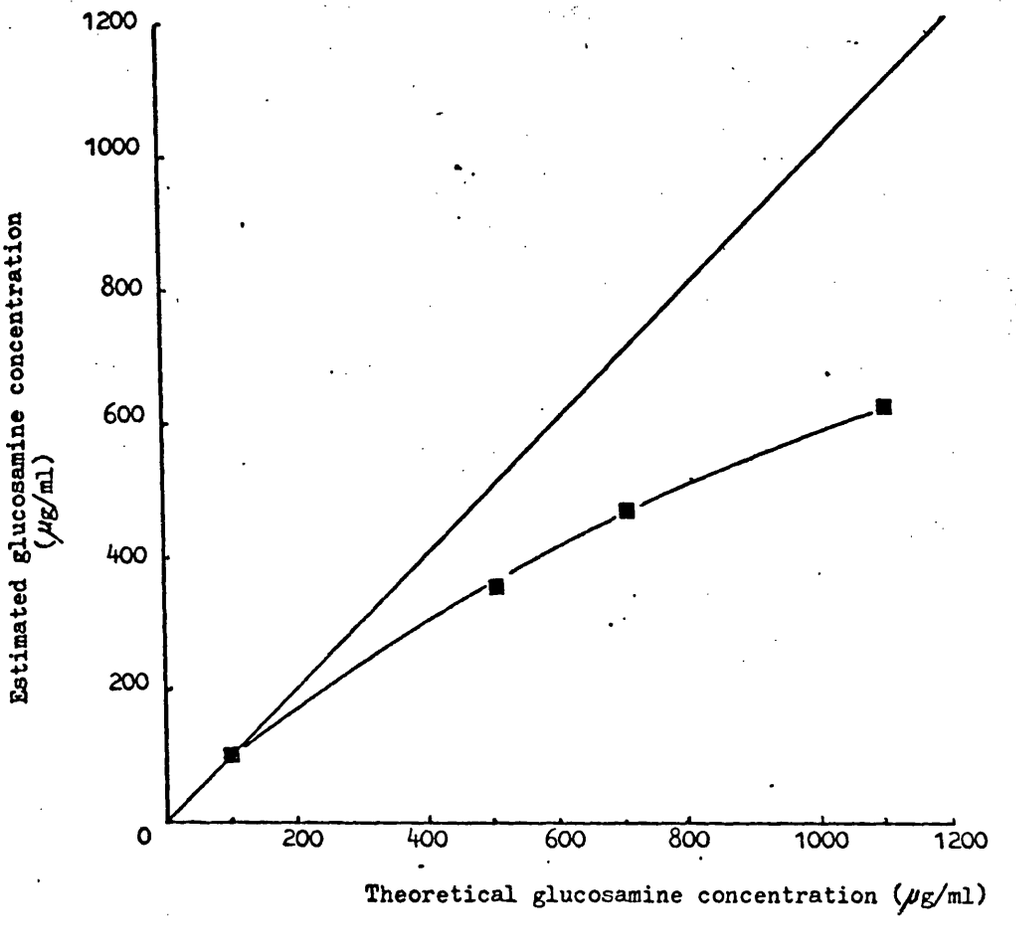


Fig 10a The estimated concentration of glucosamine added to a hay hydrolysate using the Morgan-Elson method.

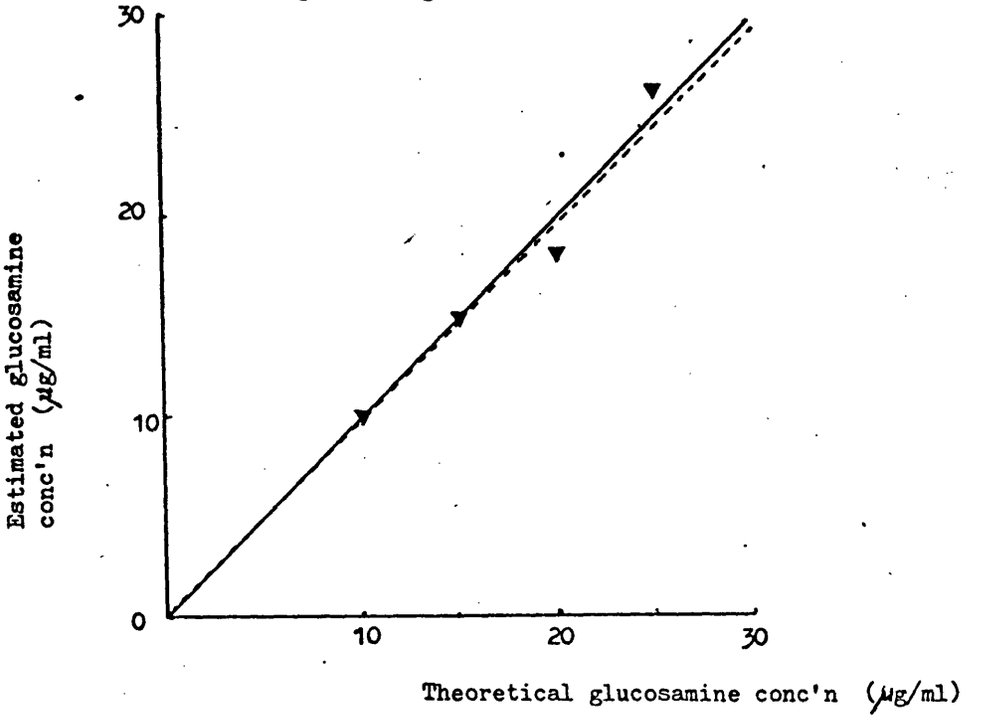


Fig 10b The estimated concentration of glucosamine added to a hay hydrolysate using the Tsuji method.

work because its use made the estimation of glucosamine too time consuming when dealing with large numbers of hay samples and the Tsuji method was considered sufficiently accurate for estimating the glucosamine content of such an inhomogenous material as hay.

The results in Fig 11 show the relationship between the total fungal spore counts and the glucosamine levels in hay taken from a deteriorated bale, a dusty bale and a clean bale. It can be seen the estimated glucosamine content of the hay was higher when determined by the Tsuji method than when determined by the Morgan - Elson method.

The fungal spore counts shown in Fig 11 were statistically analysed by analysis of variance as were the glucosamine levels. The total fungal spore counts were significantly higher ($P < 0.05$) for the deteriorated hay than for the dusty hay, which in turn were higher than for the clean hay. The glucosamine level as determined by the Morgan - Elson method, showed no difference between the deteriorated and dusty hay although both were significantly higher ($P < .05$) than for the clean hay. However, when considering the Tsuji determinations of the deteriorated hay, glucosamine levels were higher than the dusty hay glucosamine levels which in turn were higher than the glucosamine levels of the clean hay.

The results in Fig 11 additionally show that the relationships between the glucosamine levels and the total fungal spore counts were consistent, although large differences in spore counts corresponded to small differences in glucosamine levels. This was probably because fungal mycelium growing in hay could produce large numbers of spores without any significant increase in its weight.

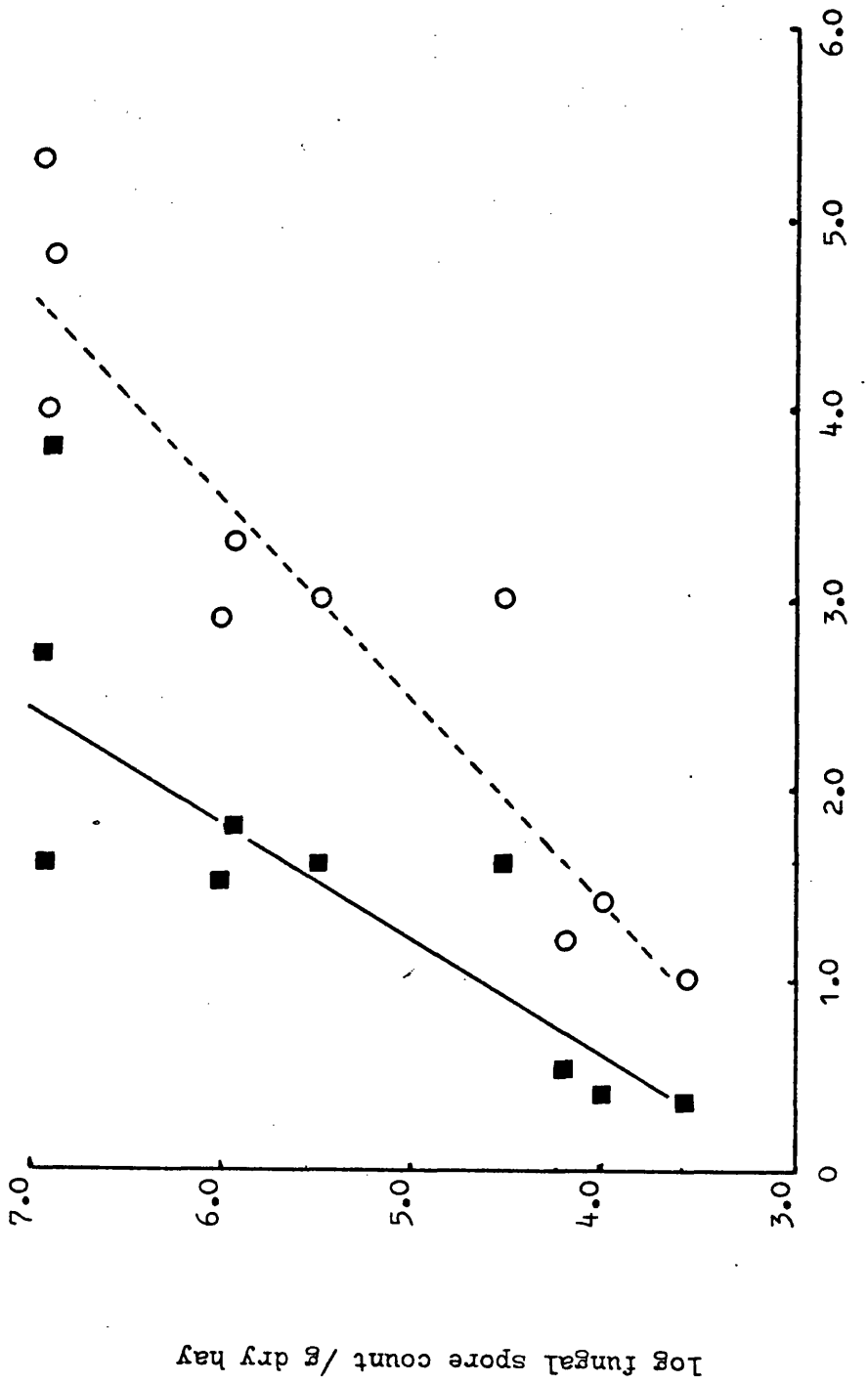


Fig 11 The relationship between the total fungal spore count of hay and the estimated glucosamine level (■) using the Morgan-Elson method (O) Using the Tsuji method.

Further relationships between the total fungal spore counts and the estimated glucosamine levels of various hay samples are shown later in this work.

ii) D.A.P.A. estimation

The possible loss of the D.A.P.A. in the hay hydrolysate, by its purification and separation from proline procedures previously described, was estimated by adding various known quantities of D.A.P.A. to 20 mls of hay hydrolysate which had been prepared for evaporation over a steam bath. The D.A.P.A. was then purified and estimated and the results obtained are shown in Fig 12.

The recovery of the added D.A.P.A. was always over 90% but tended to decrease when larger quantities of D.A.P.A. had been added to the hydrolysates. Why this should have been was not certain, but it may have been due to the column being overloaded.

It was considered possible that the regeneration of the Amberlite 120 columns with 0.2N sodium hydroxide might have affected their efficiency at purifying D.A.P.A., and therefore a series of solutions of varying known D.A.P.A. and proline concentrations were run through freshly prepared columns. The columns were then regenerated and similar solutions were passed through. The measured D.A.P.A. and proline concentrations of all the eluents are summarised in fig 13, and they suggest that the regeneration procedure did not affect the efficiency of the columns.

iii) The determination of glucosamine levels of pure fungal cultures

One of the inherent inaccuracies in determining the fungal population

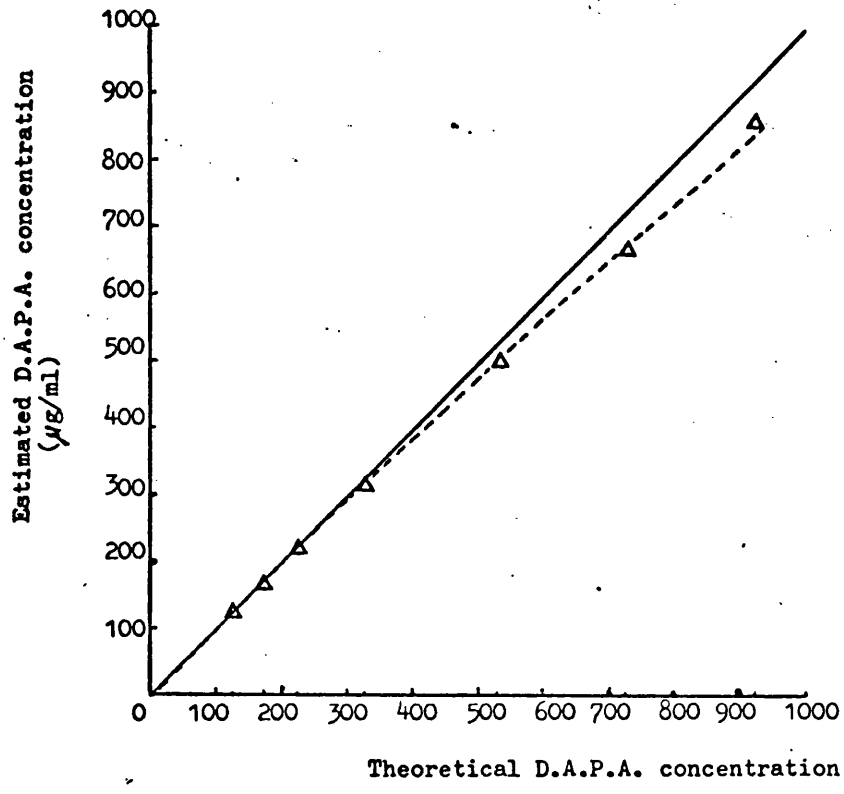


Fig 12 The estimated concentrated of diaminopimelic acid (D.A.P.A.) added to a hay hydrolysate.

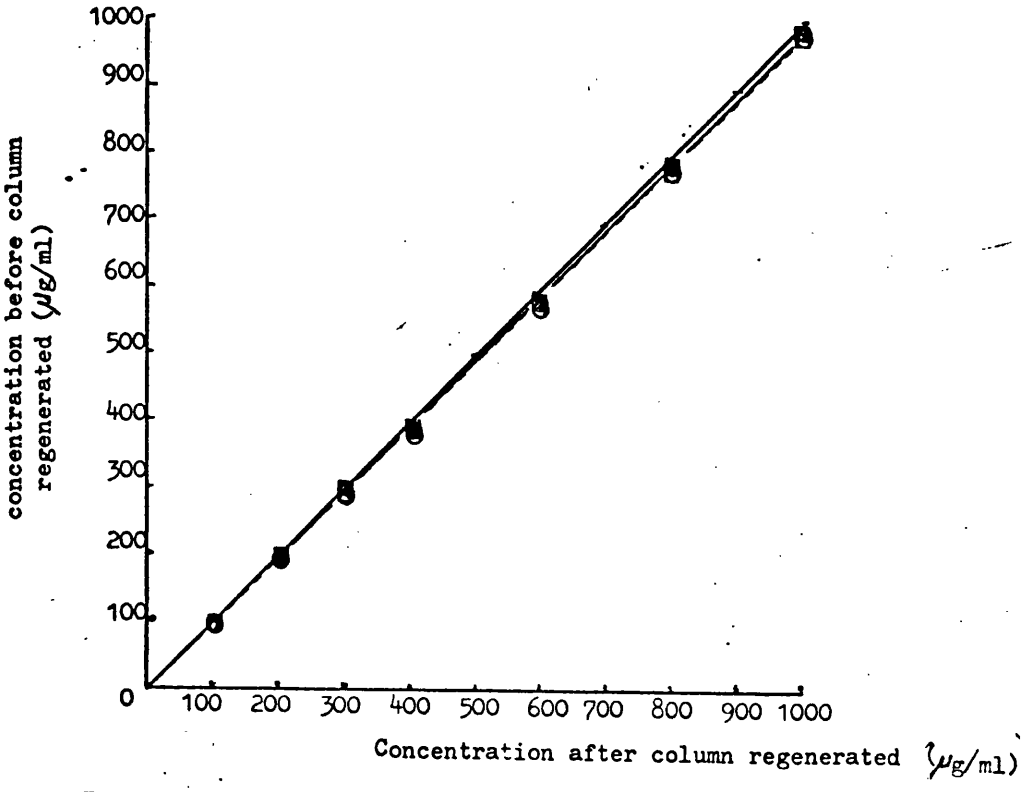


Fig 13 The effect of regenerating the Amberlite column on recovery (■) D.A.P.A. (O) Proline.

of hay by chitin estimation, was that many factors, including the growth stage of the fungus, nutrient availability and variation in the chitin content of different fungal species, would affect the percentage of the fungal dry weight that consisted of chitin (Philips 1974).

In order to obtain an estimation of the variation in the glucosamine content of several fungal species which commonly occur in deteriorated hay, the fungi were grown in pure cultures and were analysed for their glucosamine content as described earlier.

The results (Table 10) show a variation in the percentage of the dry weight of the fungi as glucosamine from 4.8% (Cladosporium herbarum) to 20.7% (a mesophilic Mucor sp.) which is an approximately four fold variation. When hay deteriorates a fungal succession occurs (Gregory et al 1963b), therefore these results suggest that the relationship between the fungal mycelium content of hay and the glucosamine content of hay would alter during deterioration, because the dominant fungal species change during deterioration.

iv) The determination of the D.A.P.A. levels of pure bacterial and actinomycete cultures

The results in Table 10 show the extent to which the glucosamine content of fungi varied between different species, therefore experiments were carried out to determine whether similar variations occurred in the D.A.P.A. levels of different bacterial and actinomycete species which commonly occur in deteriorated hay. The results are summarised in Tables 11a and 11b.

The D.A.P.A. content of the actinomycetes ranged from 0.04% of

Fungal spp	Incubation temperature	% dry weight * as glucosamine
Absidia ramosa	45°C	19.2
Aspergillus candidus	25°C	13.2
" fumigatus	45°C	14.1
" glaucus spp	25°C	11.3
" nidulans	45°C	12.4
Cladosporium herbarum	25°C	4.8
Fusarium spp	25°C	7.9
Mucor pusillus	45°C	18.4
Mucor spp	25°C	20.7
Penicillium spp	25°C	8.2
Talaromyces dupontii	45°C	7.6
Thermomyces lanuginosa	45°C	10.4
" stellata	45°C	10.7

Table 10 - The glucosamine content of fungi isolated from deteriorated hay.

* Glucosamine was estimated by the colourimetric method of Tsuji et al (1969).

Actinomycete spp	Incubation temp.	% dry weight as D.A.P.A.
Micropolyspora faeni	60°C	0.15
Thermoactinomyces glaucus	37°C	0.13
" vulgaris	60°C	0.18
Grey streptomyces isolate (1)	37°C	0.11
White " " (1)	37°C	0.08
" " " (2)	37°C	0.04
Grey " " (2)	25°C	0.04
" " " (3)	25°C	0.10
White " " (3)	25°C	0.05
" " " (4)	25°C	0.08

Table 11a- The Diaminopimelic acid (D.A.P.A.) content of actinomycetes isolated from deteriorated hay.

Bacterial spp	Incubation temperature	% dry weight as D.A.P.A.
Bacillus licheniformis	37°C	0.35
Bacillus spp isolate (1)	60°C	0.22
" " " (2)	25°C	0.28
lactobacillus spp isolate (1)	37°C	0.42
" " " (2)	25°C	0.38
Micrococus spp isolate (1)	37°C	0.08
" " " (2)	25°C	0.12
Pseudomonas spp isolate (1)	25°C	0.27
" " " (2)	25°C	0.32

Table 11b- The D.A.P.A. content of bacterial species isolated from deteriorated hay.

the dry weight (white streptomycete isolate 2 and grey streptomycete isolate 2) to 0.18% of the dry weight (Thermoactinomyces vulgaris), this being a 4.5 fold variation. The range for the bacteria was from 0.08% (Micrococus spp isolate (2) to 0.42% (Lactobacillus spp isolate (1)) which was an approximately five fold variation and considering the actinomycetes and bacteria as a whole, a ten fold variation occurred.

Overall the bacteria appeared to contain a higher proportion of D.A.P.A. than the actinomycetes with Lactobacilli spp consisting of the highest D.A.P.A. levels and Micrococci spp the lowest, the latter containing similar levels to the actinomycetes. Considering the actinomycetes, the streptomycete spp contained lower levels of D.A.P.A. than Micropolyspora faeni and the Thermoactinomyces spp.

These variations would lead to inaccuracies in estimating the actinomycete and bacterial content of hay by D.A.P.A. level estimations, because it seems likely that, as with the fungi, a microbial succession would occur in deteriorating hay.

c) Determination of the moisture content of hay

The commonest method in the literature for determining the moisture content of hay, was to dry a known weight of damp hay to constant weight at 100°C and then to reweigh the hay. It was considered possible that volatile compounds which occur in hay, especially deteriorated hay, may have been evaporated from hay at 100°C which would have yielded false moisture estimations.

In order to determine the effect of the drying temperature on the estimated moisture content of hay, clean and deteriorated hay samples were dried to constant weight at 60°C and then at ten degree intervals up to a temperature of 140°C. The results (fig 14) suggest that 80°C was the maximum drying temperature which should have been employed, because at higher temperatures other components appeared to have been evaporated from the hay, assuming all the water was evaporated from the hay at 60°C. There was the possibility that a small proportion of the water in the hay was chemically bound and that temperatures higher than 80°C were needed to evaporate this water, however, this latter theory was considered less likely than the former.

To determine a time which would ensure thorough drying of hay samples below 100g wet weight, at 80°C, hay samples containing approximately 50% moisture and weighing 100g were placed in an oven at 80°C and weighed regularly. The results (fig 15) show a drying time of between 24 and 32 hours was required to attain constant weight at 80°C, and therefore, throughout this work, when the moisture content of hay was determined care was taken to ensure that the wet weights of the samples did not exceed 100g and they were dried for a period of not less than 48 hours. These measures were considered sufficient to

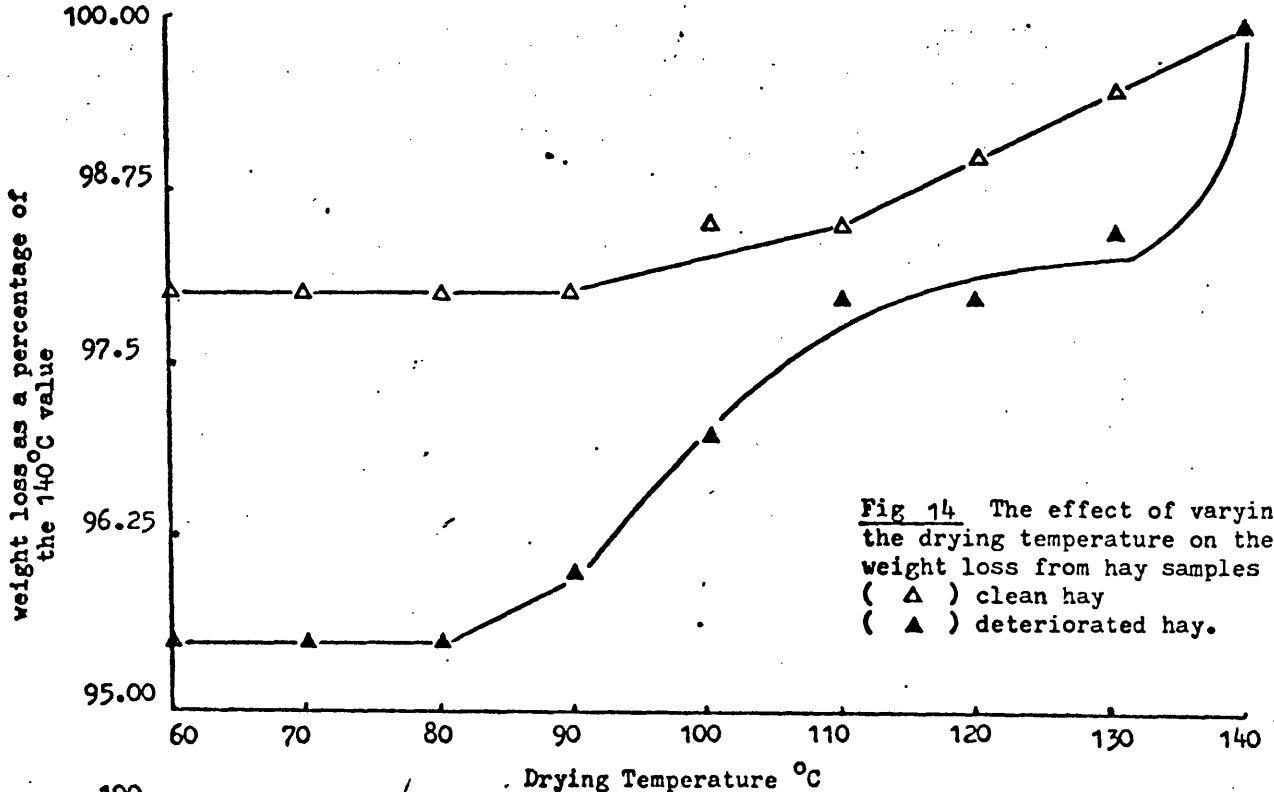


Fig 14 The effect of varying the drying temperature on the weight loss from hay samples
(△) clean hay
(▲) deteriorated hay.

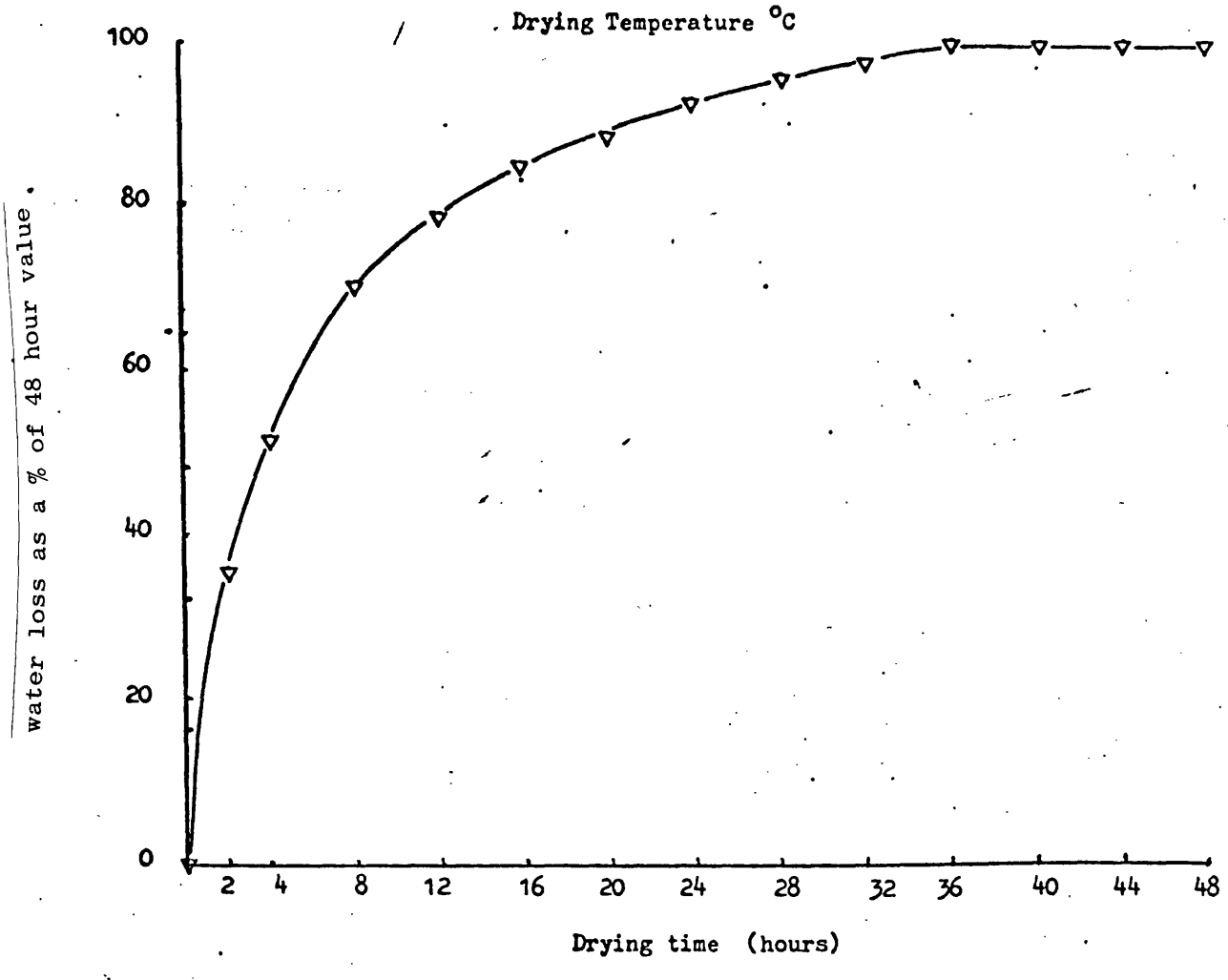


Fig 15 The rate of water loss from hay samples heated at 80°C.

ensure thorough drying of the samples.

It was therefore assumed that oven drying at 80°C was an accurate method for determining hay moisture levels, however, when it was necessary to estimate the moisture content of hay lying in the field, rapid moisture determinations were required.

Drying hay samples, taken from the field, under Infra-red lamps was experimented with, however, it was found that only small and therefore unrepresentative samples could be used, and that the procedure required too much time.

An accurate moisture meter was considered the ideal solution, however, of the several meters tried, nearly all gave inaccurate moisture levels when checked against oven drying results, especially with hays which contained more than 30% water. There was, however, one moisture meter, which was an experimental one, produced by R.D.S. Ltd and which determined the moisture content of hay samples by measuring their electrical capacitance and conductance, that gave moisture level determinations in close agreement with those obtained by oven drying (Fig 16). These results were obtained by removing twelve samples, each weighing between 50g and 100g (wet weight) from a bale of hay immediately after baling. The moisture levels of these samples were estimated by the use of the R.D.S. meter and then by oven drying.

These results (fig 16) show, firstly that the moisture meter water level determinations were similar to oven drying results and secondly, the variation in the moisture levels which occurred within the bale

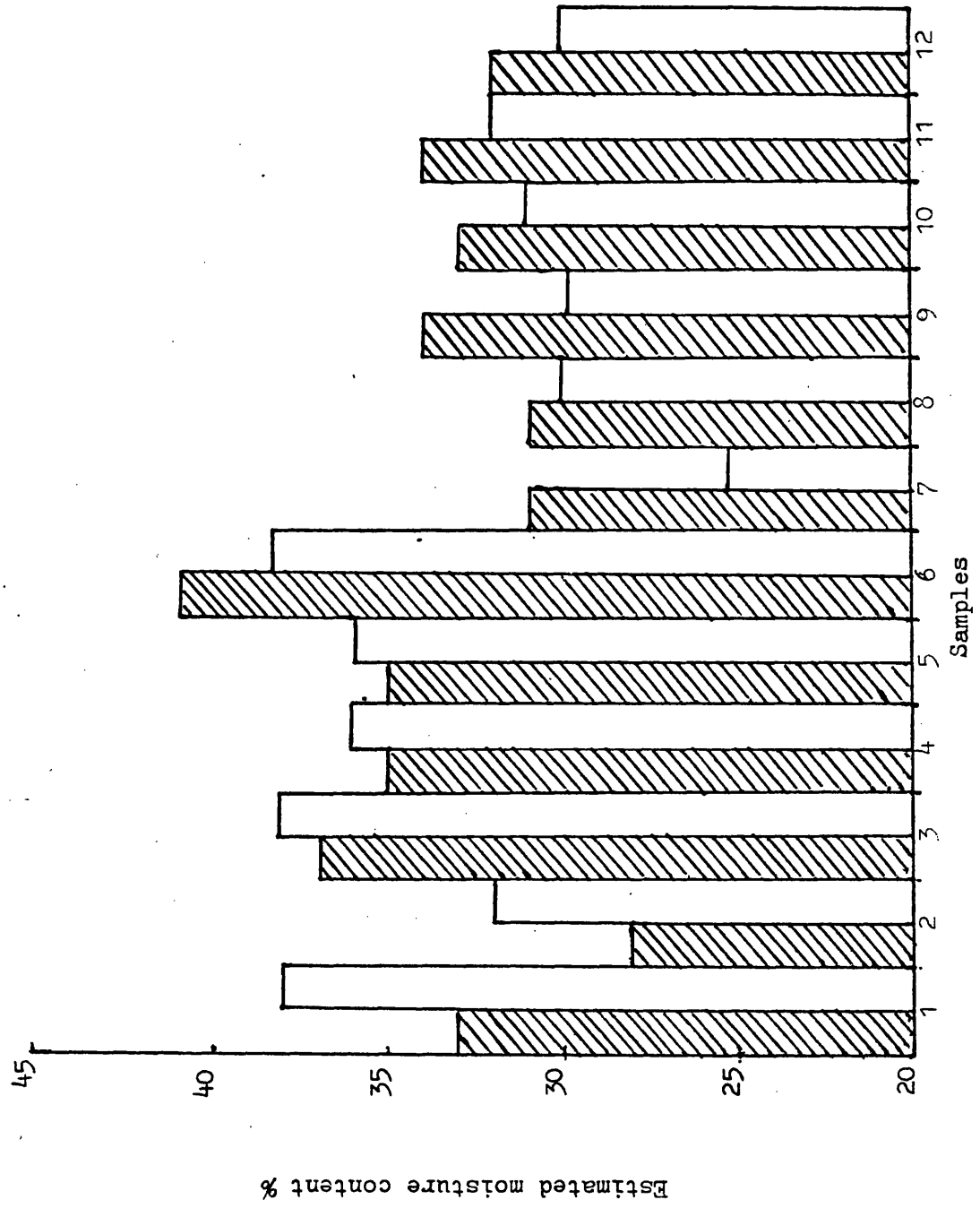


Fig 16 Moisture level determinations on twelve samples taken from one bale of hay.

▨ Using the R.D.S. moisture meter ☐ By oven drying at 80°C.

of hay. It was considered that the application rates of preservatives would have to be sufficient to prevent deterioration of the dampest patches of hay.

d) Determination of organic acids applied to hay

Estimates of the organic acid levels on treated hay were needed to calculate, firstly the acid distribution within treated bales and secondly their persistence after application.

i) Volatile fatty acids

The initial step in estimating the volatile fatty acid levels was to extract these acids from the hay. Early in this work the method used was to soak the hay samples in 0.6N H_2SO_4 for seven days and then to estimate the volatile fatty acid levels in the H_2SO_4 by the gas liquid chromatographic method described earlier. The recovery of volatile fatty acids from hay was poor using this method (Table 12) being between 60% and 90%, therefore the preferred method, of heating the hay in kilner jars with a sodium hydroxide solution, which gave almost complete volatile fatty acid recovery (Table 12), was adopted for later results.

Weight of propionic acid added to 12.5g of hay	Weight of n-butyric acid added to 12.5g of hay	% propionic acid recovered by acid soaking	% n-butyric acid recovered by acid soaking	% Propionic acid recovered by heating in Kilners	% n-butyric acid recovered by heating in Kilners
0.125g	-	70%	-	97.5%	-
0.250g	-	64%	-	98.0%	-
0.125g	0.250g	72%	90%	100.0%	100.0%

Table 12 - Comparison of the percentage extraction of volatile fatty acids added to hay by soaking in 0.6N H_2SO_4 for 7 days or by heating in Kilner jars at 90°C for 5 days.

The kilner jars were heated at 90°C because the higher the temperature the more rapid the volatile fatty acid evaporation from the hay, however, at 100°C too much water evaporated from the sodium hydroxide solution, therefore 90°C appeared to be the most suitable compromise temperature.

The volatile fatty acids were estimated after extraction from the hay by gas liquid chromatography as previously described. Fig 17a shows a typical trace obtained from a kilner jar extraction from hay containing a high level (0.6%) of propionic acid, the n-butyric acid being added as an internal standard. Acetic acid occurred naturally in hay at significant levels and the gas liquid chromatography trace in fig 17b shows how the acetic acid peak could mask the propionic acid peak when the extract was from hay containing a low level (0.006%) of propionic acid. This interference made the accurate estimation of propionic acid levels less than 0.02% on hay, difficult.

Fig 18 shows a typical standard curve which was used to relate the ratio of propionic acid and n-butyric acid peak heights to the weight ratio of the two acids, so that when a known amount of n-butyric acid was added to the hay in the kilner jar, using this standard curve and the propionic acid peak height, the propionic acid level on the hay sample could be determined. The actual slope of this graph was found to vary from day to day and also with the gas liquid chromatogram attenuation employed.

ii) Sorbic acid

Sorbic acid could not be extracted from hay by heating in Kilner

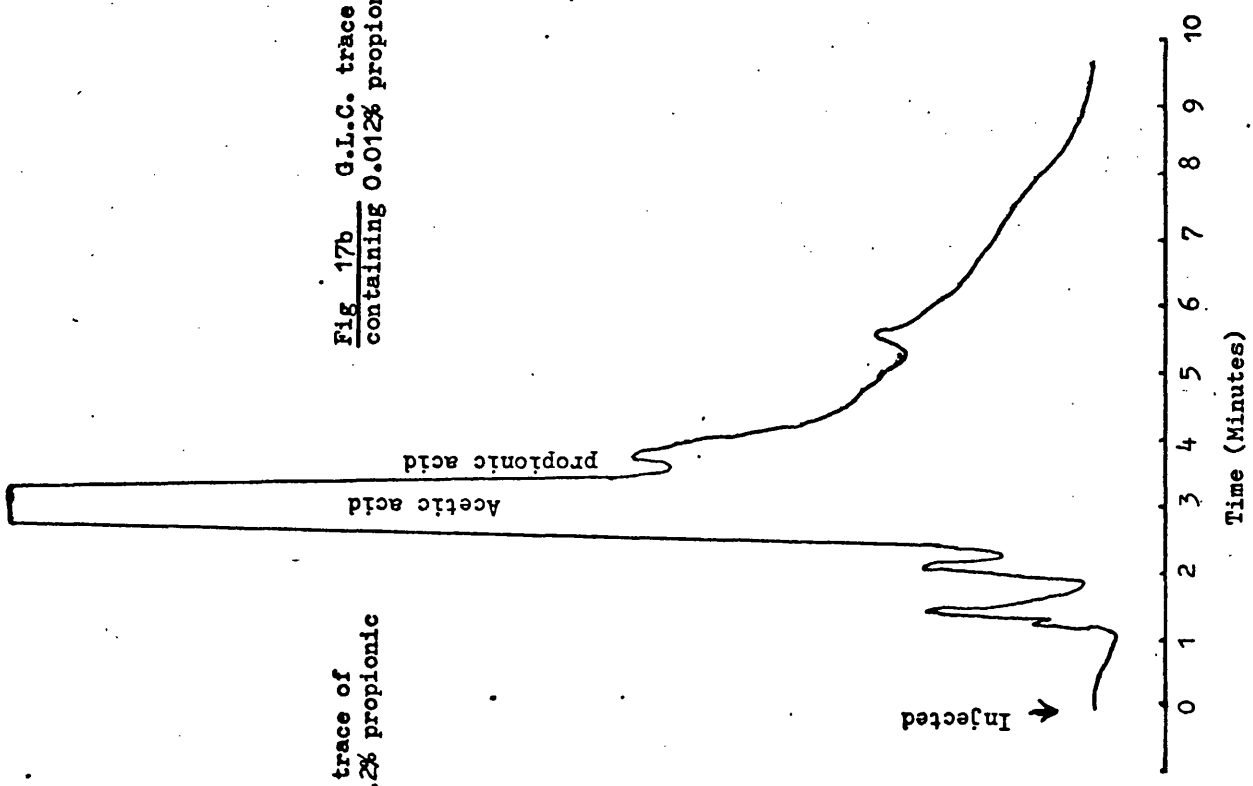


Fig 17b G.L.C. trace of sample containing 0.012% propionic acid.

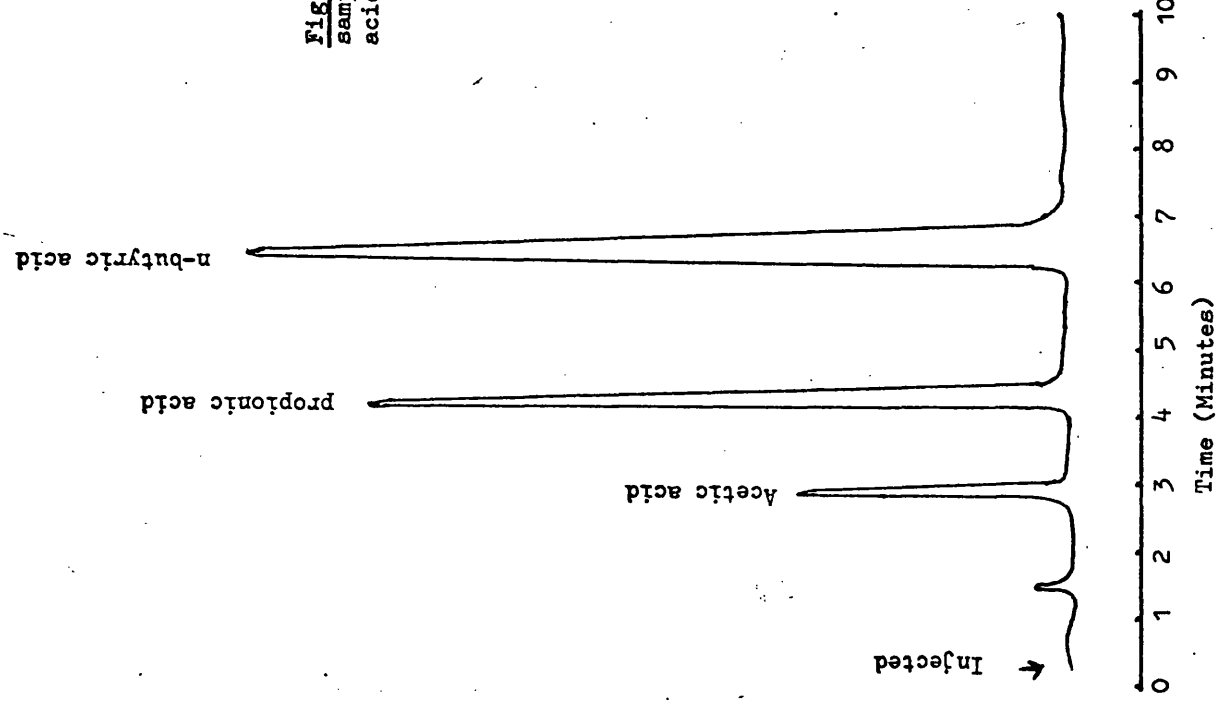


Fig 17a G.L.C. trace of sample containing 1.2% propionic acid.

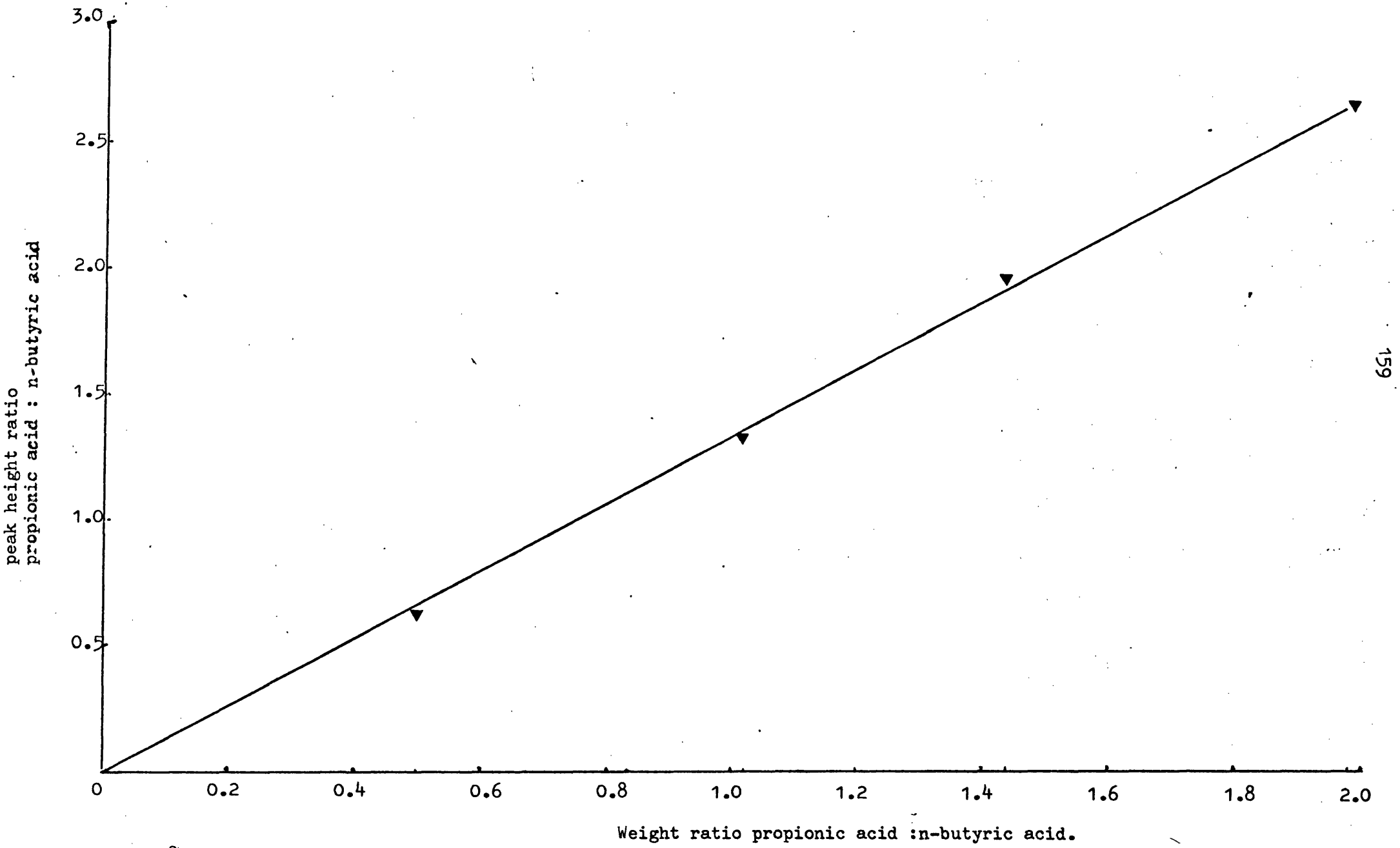


Fig 18 Relationship between G.L.C. peak height ratio and weight ratio for propionic acid : n-butyric acid mixtures.

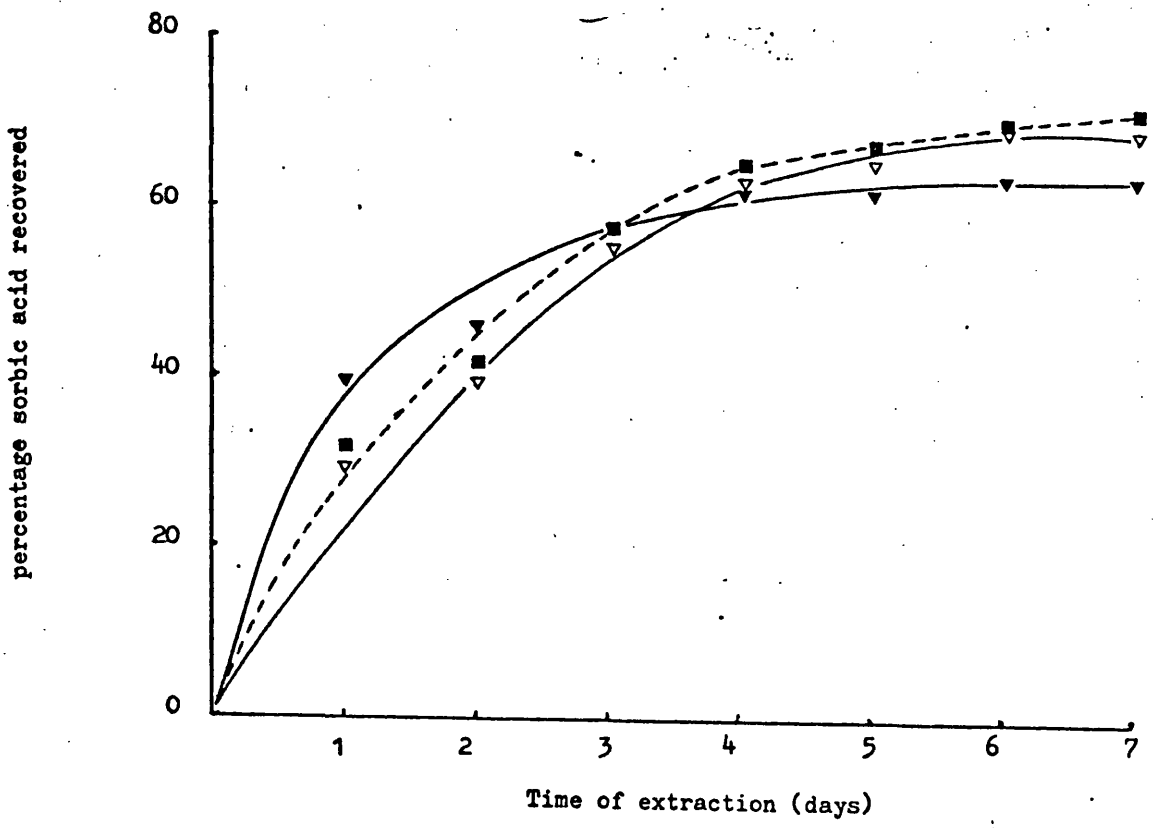


Fig 19 The extraction of added sorbic acid from hay by soaking in 0.6N H_2SO_4 (▼) 1st sample (■) 2nd sample (▽) 3rd sample.

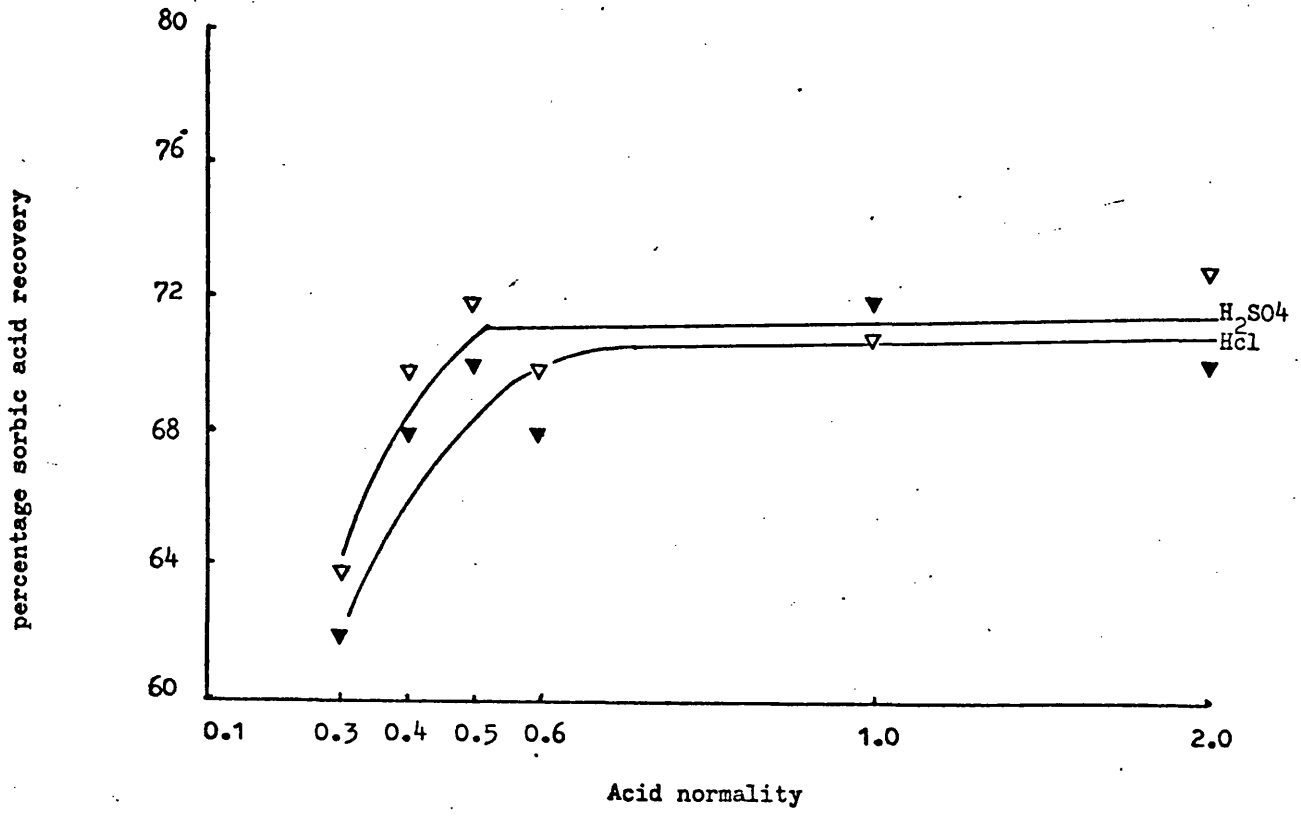


Fig 20 Effect of varying the acid strength on the recovery of sorbic acid added to hay (▽) H_2SO_4 (▼) Hcl.

jars because it is non-volatile and therefore it was extracted by soaking the hay in 0.6N H_2SO_4 for seven days. The results in fig 19 show that between 60% and 80% of sorbic acid, which had been added to hay as a 10% solution in propionic acid, was transferred to the aqueous sulphuric acid after an extraction period of seven days. The results also suggest that a longer period of extraction would not have improved the recovery. It was considered that this poor recovery could have been due to the sorbic acid reacting with components in the hay, therefore the use of more concentrated mineral acid solutions were tried in the hope they would have improved the sorbic acid recovery. Unfortunately the results obtained (fig 20) show that an increase in the acid strength above 0.6N did not increase the weight of sorbic acid extracted from the hay.

Throughout this work the sorbic acid levels were determined by soaking the hay in 0.6N H_2SO_4 and estimating the sorbic acid levels in the sulphuric acid colourimetrically as previously described. It was considered that there may have been interference to the colourimetric determinations, from components in the hay and therefore treated hay extracts were scanned using a Pye Unicam SP1500, the sorbic acid peaks occurring at 532 mμ. Fig 21a shows how, with an extract from hay containing a high level of sorbic acid (.35%), the sorbic acid peak is clear and suffers from little intereference, however, with an extract from hay with a low sorbic acid level (.02%) as shown in fig 21b, other components in the hay interfered with the sorbic acid peak and therefore hay extracts containing less than 50 p.p.m of sorbic acid were scanned on the SP1500 in order to accurately estimate the sorbic acid peak size.

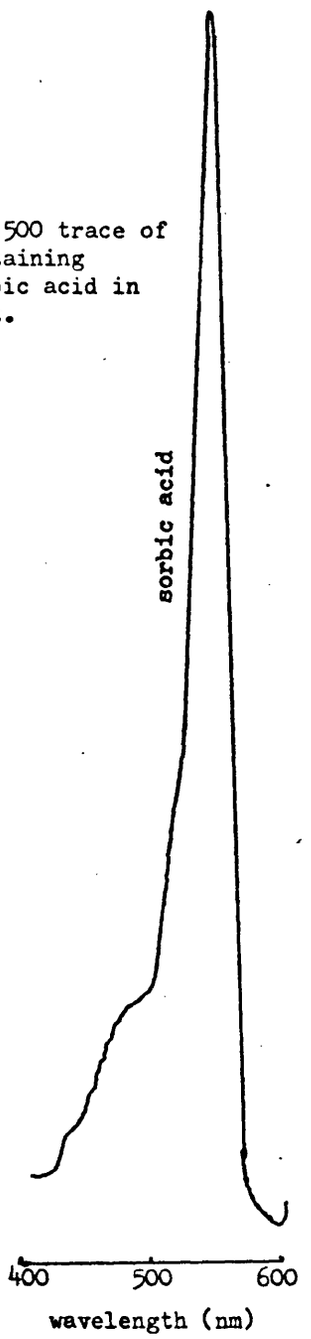


Fig 21a SP1500 trace of a sample containing 350p.p.m sorbic acid in a hay extract.

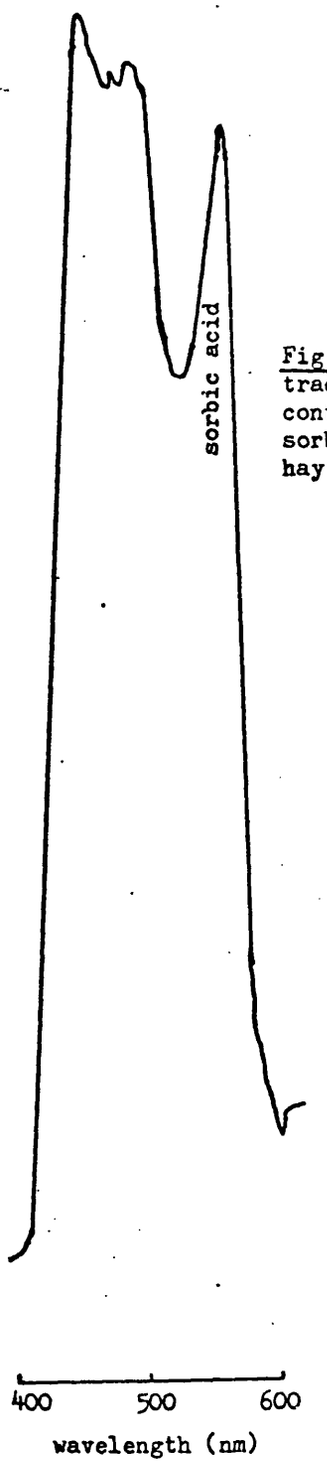


Fig 21b SP 1500 trace of a sample containing 20 p.p.m. sorbic acid in a hay extract.

e) Determination of hay p.H and its buffering capacity

As micro-organisms grow in hay they raise the p.H value (Gregory et al 1963b), therefore it was considered that the measurement of the p.H of hay could have given a rough estimation of the extent of its microbial deterioration.

The method used to measure the p.H was to blend a known weight of hay with distilled water and then to measure the p.H of the solution. Difficulties arose when large quantities of hay were blended with water because of the volume of the material, and therefore, the effect of the weight of hay blended upon the determined p.H value for the hay was examined. Hay was removed from a fresh bale and from a deteriorated bale, dried at room temperature (heating increases the p.H value of hay) and divided into ten 5g portions. Considering the clean hay, one 5g portion was blended with 500 mls of distilled water and the p.H value of the solution was measured. A second 5g portion was added, blended and the p.H was again measured. This process was repeated until the 50g of hay had been blended with the water. The procedure was then repeated with the deteriorated hay.

The results (fig 22) show that the addition of clean hay decreased the p.H value of the solution whereas the deteriorated hay samples increased the p.H value suggesting that the clean fresh hay was weakly acid whereas the deteriorated hay had become alkaline probably due to microbial metabolic products, including ammonia formed from protein breakdown. The addition of further quantities of hay became impractical because all the water had been absorbed by the 50g of hay.

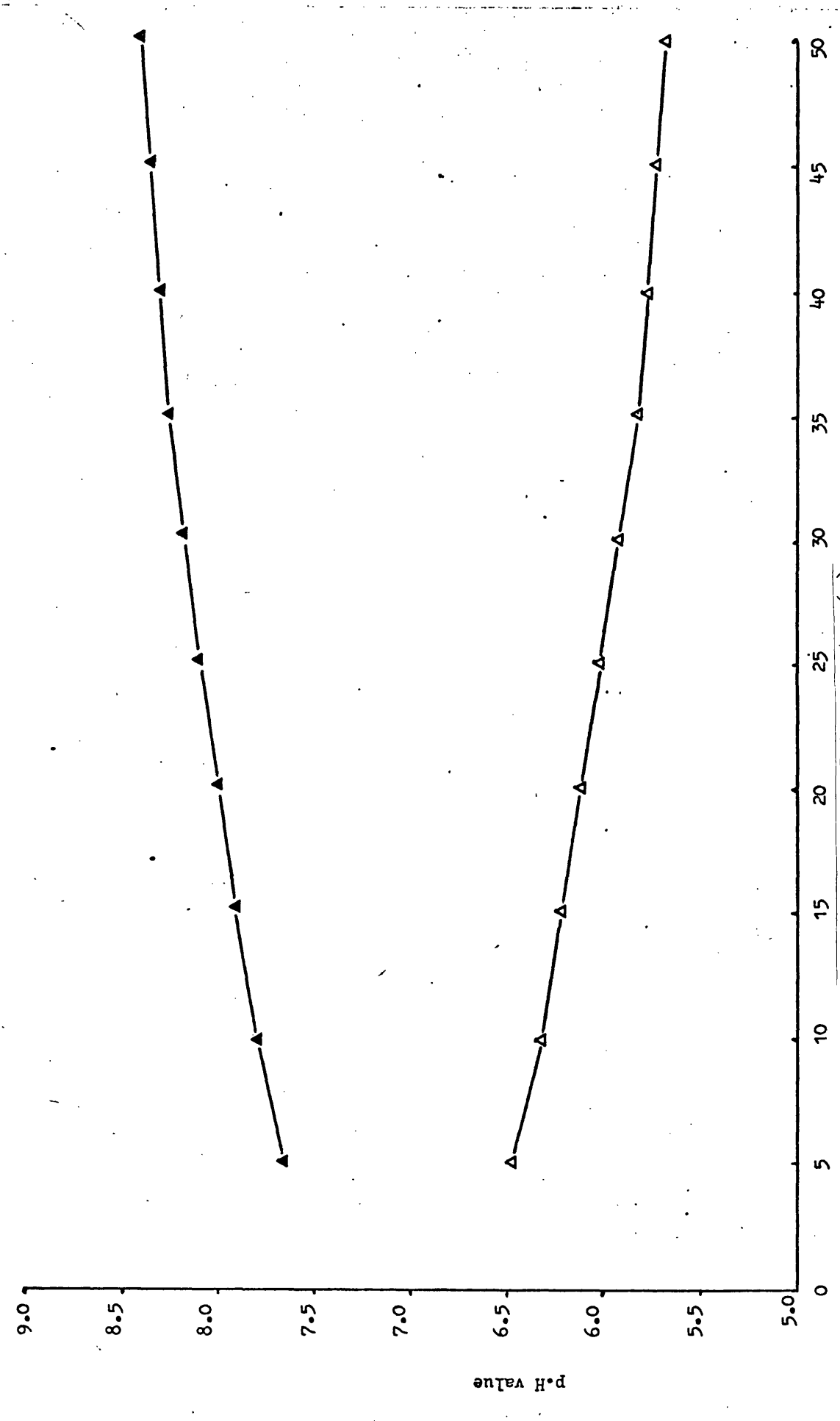


Fig 22 The effect of the hay sample weight on the estimated p.H value (Δ) clean hay (▲) deteriorated hay

In further experiments larger quantities of hay than 50g were cut into 2 to 3cm lengths and blended with 500 mls distilled water. The measurement of the p.H. of the resulting mixtures was attempted using a p.H. electrode designed to work in semi-solid media, however, the results obtained were inconsistent probably because all the hay sap had not been released as the hay could not be effectively blended.

The anti-microbial activity of many microbicides, including organic acids, increases with a decrease in p.H, therefore if an organic acid was applied to hay as a preservative it would probably be more effective if the p.H of the hay could be lowered. In order to determine whether a mineral acid lowered the p.H of hay more than propionic acid, the buffering capacity of hay was estimated against propionic and sulphuric acids as previously described.

The results in fig 23 show how the p.H of a litre of distilled water was lowered by the addition of 0.1N H_2SO_4 and 0.1N propionic acid. The sulphuric acid gave lower p.H values because of its more complete ionisation in water. When 1.0N H_2SO_4 and 1.0N propionic acid were added to 50g of hay blended in 1 litre of distilled water, the results shown in fig 24 were obtained. The addition of 10 mls of 1.0N H_2SO_4 lowered the p.H values of clean and deteriorated hay to 4.5 whereas 10 mls of 1.0N propionic acid lowered the p.H values to 5.0. Since 10 mls of 0.1N sulphuric and propionic acids lowered the p.H of water to below 4.0 (fig 23) the buffering capacity of hay is well demonstrated by the results in fig 24, which would be expected because of the many chemical components of the material.

These results also suggest that 0.98% of sulphuric acid applied

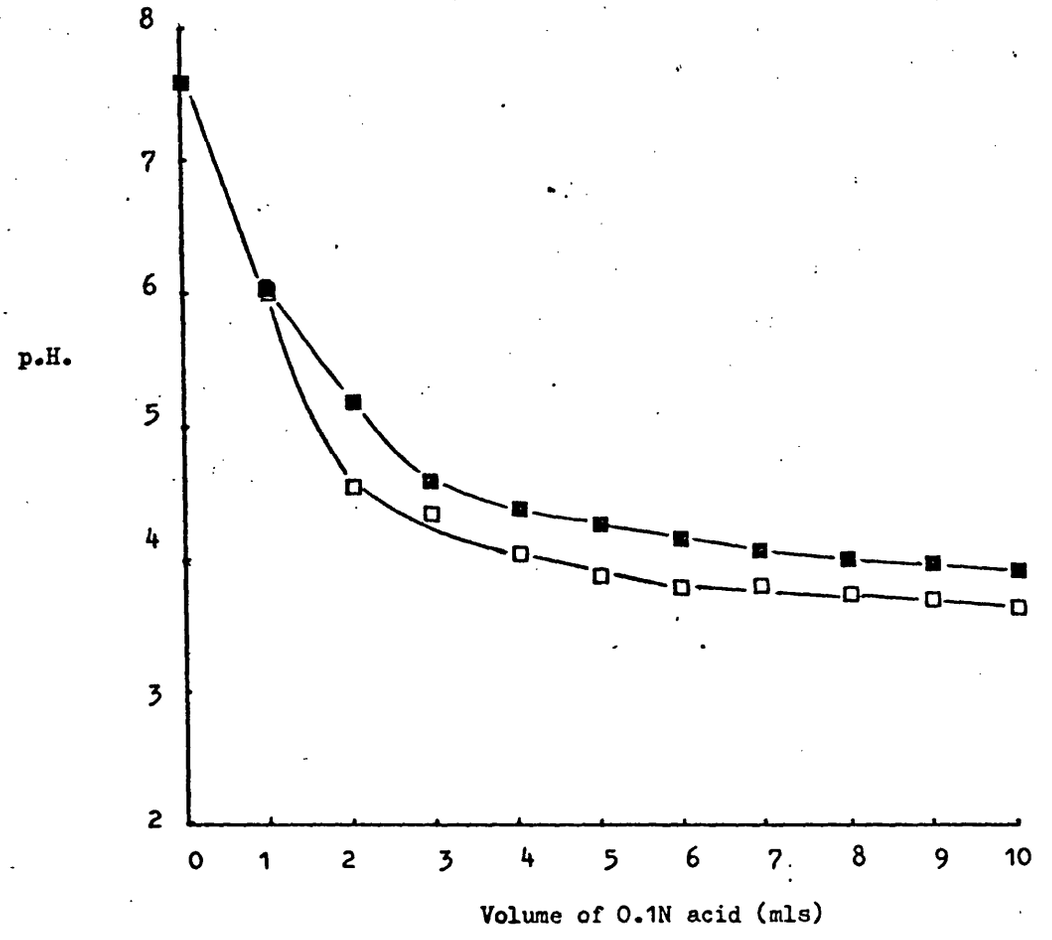


Fig 23 The effect on the p.H. of 1 litre of distilled water, of adding 0.1N acids (□) H_2SO4 (■) propionic acid.

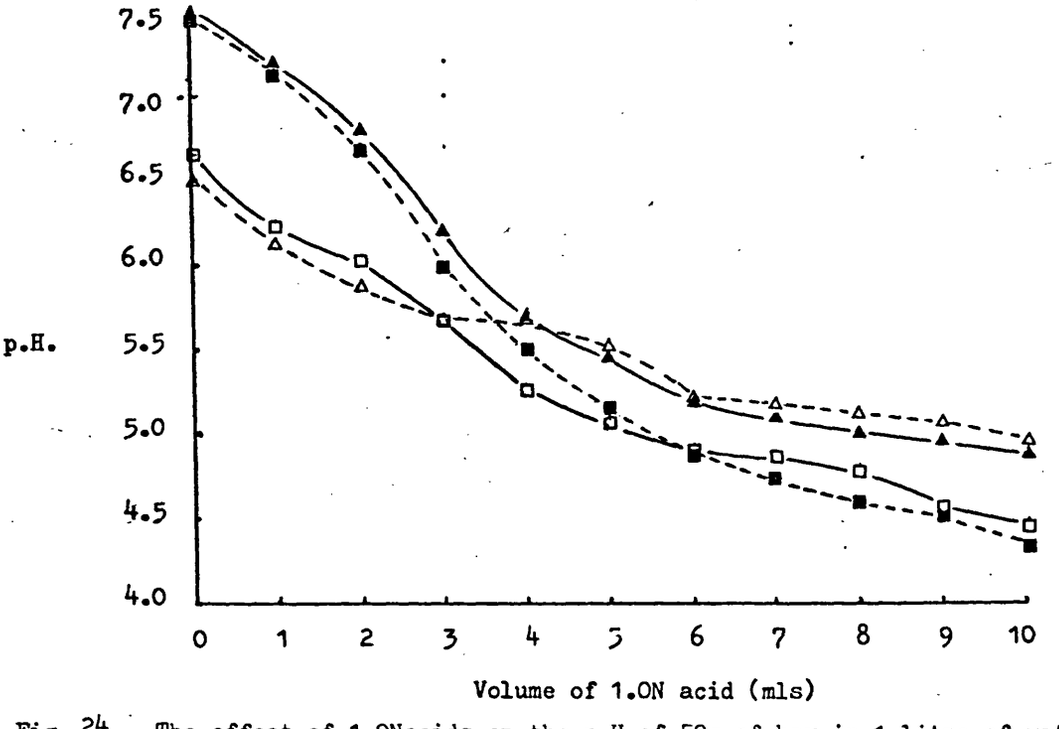


Fig 24 The effect of 1.ONacids on the p.H of 50g of hay in 1 litre of water (□) H_2SC_4 on clean hay (■) H_2SO_4 on deteriorated hay (△) propionic acid on clean hay (▲) propionic acid on deteriorated hay.

to hay, would lower the p.H to approximately 4.5, that 1.0% propionic acid on hay would lower the p.H to 5.25 and that 1.48% propionic acid on hay would lower the p.H to 5.0. The lowering of the p.H of hay would be a desirable effect not only because it would enhance the anti-microbial activity of added organic acids but it would also prevent the growth of many micro-organisms, especially the actinomycetes, directly.

F) Fluorescent dye solutions and their comparative fluorescence on hay

In order to assess the spray pattern on baled hay which was obtained using the current preservative applicators, a fluorescent dye solution was sprayed on the hay which was then baled. The bale was broken open and the hay viewed, under an ultra-violet lamp, in a dark room.

To determine which dye solution would fluoresce most clearly on hay, suspensions of the dyes in water were prepared, and firstly grass blades were dipped into the solutions and secondly small batches of hay were sprayed with the solutions, at rates of 0.5% and 1.0% The grass blades and hay were then viewed under an ultra-violet lamp in a dark room and their comparative visibilities were assessed visually.

The first fluorescent dye tested was primuline, however, a primuline solution fluoresced poorly on hay. It was also observed that the fluorescent dye was less clearly visible on fresh green hay than on older hay, and therefore it was considered possible that the main explanation, for this fluorescence quenching, was ultra-violet light absorption by chlorophylls in the hay.

In an attempt to improve the fluorescence of primuline, the

compounds tween 80, starch and polyvinyl alcohol were added to the primuline solution before it was applied to the grass or hay. The comparative strengths of fluorescence of these mixtures was then assessed visually and summarised in Table 13.

The addition of Tween 80 improved the primuline fluorescence, possibly because it permitted spreading of the dye solution over the hay surface to give a more extensive coverage. The primuline with polyvinyl alcohol gave the brightest fluorescence possibly because the dye was concentrated onto the polyvinyl alcohol granules and was therefore kept away from the quenching components in the hay. The addition of Tween 80 to the primuline and polyvinyl alcohol mixture slightly reduced fluorescence on the grass blades possibly because the wetting agent tended to reduce the attraction of the primuline for the polyvinyl alcohol granules.

The solution which fluoresced most clearly on hay was 0.2% primuline in a 1.0% polyvinyl alcohol solution in water. However, these concentrations were too high for field work applications because of the large volumes involved and were therefore reduced to 0.1% primuline in a 0.2% polyvinyl alcohol solution.

Even after the addition of polyvinyl alcohol, the primuline solutions did not fluoresce clearly after being sprayed on baled hay, partly because of their yellow fluorescence which became masked by the yellow fluorescence which naturally occurred in hay. In an attempt to find more suitable fluorescent materials, comparisons were made between a primuline and polyvinyl alcohol solution and four pigment tracer suspensions in water. The results in Table 14 show that all the

pigment tracers fluoresced more strongly on hay than the primuline - polyvinyl alcohol solution, and that their fluorescence was not improved by the addition of Tween 80 or polyvinyl alcohol. The tracer fire orange appeared to fluoresce the most strongly, neon red gave a duller fluorescence and saturn yellow, as with primuline, suffered from interference from some natural yellow fluorescence of hay.

As a result of this experiment, in the latter part of this work, a 0.5% suspension of fire orange in water was employed for the assessment of the spray patterns obtained using hay preservative applicators.

The actual spray patterns observed are described later in this report.

Solution in Distilled water	Comparative visibilities on grass blades	Comparative visibilities on fresh hay
0.1% primuline	*	+
0.2% "	**	+
0.3% "	**	
0.4% "	**	
0.5% "	**	
0.2% primuline + 0.1% Tween 80	***	
0.2% " + 0.2% " "	****	+
0.2% " + 0.4% " "	****	
0.2% " + 0.5% starch	***	
0.2% " + 0.5% " + 0.2% Tween 80	****	+
0.2% primuline + 0.5% polyvinyl alcohol	****	++
0.2% " + 1.0% " "	*****	+++
0.2% " + 2.0% " "	*****	+++
0.2% " + 1.0% " " + 0.2% Tween 80	****	
0.2% primuline in propionic acid	None	None

Table 13 - The comparative fluorescence of various primuline solutions on grass blades and hay when viewed under an ultra-violet lamp.

* and + - See table 14

Solution in Distilled water	comparative visibilities on fresh hay
0.2% primuline + 1.0% polyvinyl alcohol	+++
0.5% " + 1.0% " "	+++
0.2% Neon Red	++++
0.5% Neon Red	+++++
0.2% Fire Orange	+++++
0.5% Fire Orange	++++++
0.2% Arc chrome	+++++
0.5% Arc chrome	+++++
0.2% Satum yellow	++++
0.5% Saturn yellow	+++++
0.5% Fire Orange + 0.2% Tween 80	++++++
0.5% Fire Orange + 1.0% polyvinyl alcohol	++++++

Table 14 - The comparative fluorescence of various mixtures on fresh hay, when viewed under an ultra-violet lamp.

Symbols for Tables 13 and 14

* poor coverage
** fair coverage
*** good coverage
**** almost complete coverage
***** Complete coverage

\+ - occasional dye spot
++ - several dye spots
+++ - poor visible dye coverage
++++ - fair visible dye coverage
+++++ - good visible dye coverage
++++++ - Very good visible dye coverage

SECTION B - THE SCREENING OF ANTIMICROBIAL COMPOUNDS

a) Organic acid activity against pure cultures in agar media

It has been shown earlier in this work that short chain volatile fatty acids and possibly sorbic acid appeared to have the best potential as hay preservatives. Therefore, pure cultures of a selection of micro-organisms which commonly occur in deteriorated hay were tested against the organic acids, acetic, propionic, n-butyric and sorbic and against mixtures of these acids consisting of acetic: sorbic 9:1 (W/W) propionic: sorbic (9:1) (W/W) n-butyric : sorbic (9:1)(W/W) and propionic: n-butyric: sorbic 45:45:10 (W/W/W). These acids were added to chemical agar media buffered at p.H values of 5, 6, 7 and 8, at various concentrations and after inoculation and incubation the minimum totally inhibitory levels were noted and summarised in Tables 15, 16, 17 and 18.

Where sorbic acid alone was included, it was firstly dissolved as a 10% solution (W/W) in ethanol, which was added to the media, this then being steamed for 20 minutes to evaporate off the ethanol. Where sorbic acid was added along with a volatile fatty acid it was dissolved in the organic acid at the concentrations described above, sterilised by membrane filtration and finally added to the media.

The results show that all the organic acids tested, inhibited the growth of the micro-organisms at approximately the same levels. Sorbic acid demonstrated the strongest anti-microbial activity, n-butyric was more effective than propionic and which in turn was more inhibitory than acetic acid. An exception to this rule occurred with the actinomycetes

where sorbic acid tended to be less effective than propionic and n-butyric acids. The addition of sorbic acid to the volatile fatty acids increased their anti-microbial activity, especially at low p.H values, but these mixtures were not as effective as the sorbic acid alone, although this latter observation was not true in the case of the actinomycetes.

Considering the fungi tested, Aspergillus fumigatus showed the most resistance and the phycomycetes along with Thermomyces lanuginosa were the least resistant. In the case of the bacteria and actinomycetes the thermophiles (incubated at 60°C) showed less resistance than the mesophiles although this may have been due to the higher incubation temperature increasing the activity of the acids. Overall, Lactobacilli were the most resistant micro-organism tested.

Micro-organisms	Incubation temperature	Minimum inhibitory concentration %							
		Acetic acid	propionic acid	n-butyric acid	sorbic acid	Acetic: sorbic	propionic: sorbic	n-butyric :sorbic	propionic: n-butyric: sorbic
FUNGI									
Absidia ramosa	45°C	0.2	0.06	0.04	0.04	0.1	0.04	0.02	0.02
Aspergillus fumigatus	45°C	0.4	0.1	0.04	0.04	0.2	0.06	0.04	0.04
" glaucus spp	45°C	0.2	0.1	0.04	0.02	0.1	0.04	0.02	0.02
Mucor pusillus	45°C	0.2	0.04	0.02	0.02	0.1	0.02	0.02	0.02
Thermomyces lanuginosa	45°C	0.08	0.02	0.02	0.01	0.02	0.02	0.01	0.01
Aspergillus spp (1)	25°C	0.2	0.1	0.04	0.04	0.1	0.06	0.04	0.04
Aspergillus spp (2)	25°C	0.2	0.08	0.04	0.02	0.1	0.04	0.04	0.04
Aspergillus candidus	25°C	0.2	0.1	0.04	0.04	0.1	0.06	0.04	0.04
Mucor spp	25°C	0.2	0.06	0.02	0.04	0.1	0.04	0.02	0.02
Penicillium spp	25°C	0.2	0.1	0.04	0.04	0.1	0.06	0.04	0.04
ACTINOMYCETES									
Micropolyspora faeni	60°C	0.1	0.02	0.02	0.1	0.08	0.04	0.02	0.02
Thermoactinomyces vulgaris	60°C	0.1	0.02	0.02	0.08	0.06	0.06	0.04	0.02
" glaucus	37°C	0.2	0.04	0.06	0.08	0.1	0.06	0.06	0.06
White streptomyces (1)	37°C	0.2	0.04	0.06	0.06	0.1	0.06	0.06	0.06
" " (2)	37°C	0.2	0.04	0.08	0.1	0.1	0.08	0.08	0.06
" " (3)	25°C	0.2	0.06	0.08	0.08	0.1	0.08	0.08	0.06
Grey "	25°C	0.2	0.04	0.04	0.1	0.1	0.08	0.06	0.04
BACTERIA									
Bacillus spp (1)	60°C	0.06	0.04	0.04	0.01	0.04	0.04	0.02	0,02
Bacillus spp (2)	60°C	0.06	0.04	0.04	0.01	0.04	0.04	0.02	0.02
Bacillus licheniformis	37°C	0.08	0.06	0.06	0.02	0.06	0.06	0.06	0.06
Bacillus spp (3)	37°C	0.06	0.06	0.04	0.02	0.06	0.04	0.04	0.04
Lactobacillus spp (1)	37°C	0.6	0.4	0.2	0.1	0.4	0.2	0.2	0.2
Bacillus spp (4)	25°C	0.06	0.04	0.04	0.02	0.06	0.04	0.04	0.04
Lactobacillus spp (2)	25°C	0.8	0.6	0.6	0.2	0.6	0.6	0.4	0.4
Micrococcus spp	25°C	0.1	0.08	0.06	0.02	0.08	0.06	0.06	0.06
Pseudomonas spp	25°C	0.2	0.1	0.08	0.04	0.1	0.08	0.08	0.08

Table 15 - The minimum inhibitory concentration of organic acids against micro-organisms occurring in hay, at p.H 5.0.

Acetic: sorbic = Acetic acid: sorbic acid 9:1 w/w

Propionic: sorbic = propionic acid: sorbic acid 9:1 w/w

n-butyric: sorbic = n-butyric acid: sorbic acid 9:1 w/w

propionic: n-butyric: sorbic = propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)

Micro-organism	Incubation temperature	Minimum inhibitory concentration %							
		Acetic acid	propionic acid	n-butyric acid	sorbic acid	Acetic: sorbic	propionic: sorbic	n-butyric: sorbic	propionic: n-butyric: sorbic
FUNGI									
Absidia ramosa	45°C	0.4	0.2	0.2	0.1	0.2	0.1	0.2	0.2
Aspergillus fumigatus	45°C	0.6	0.4	0.2	0.1	0.4	0.2	0.2	0.2
" glaucus spp	45°C	0.4	0.4	0.2	0.06	0.2	0.1	0.2	0.2
Mucor pusillus	45°C	0.4	0.2	0.2	0.1	0.2	0.1	0.2	0.2
Thermomyces lanuginosa	45°C	0.2	0.1	0.1	0.06	0.1	0.08	0.08	0.1
Aspergillus spp (1)	25°C	0.4	0.4	0.2	0.06	0.2	0.1	0.2	0.2
" spp (2)	25°C	0.4	0.4	0.2	0.06	0.2	0.2	0.2	0.2
" candidus	25°C	0.4	0.4	0.2	0.1	0.2	0.2	0.2	0.2
Mucor spp	25°C	0.4	0.2	0.2	0.1	0.2	0.1	0.2	0.2
Penicillium spp	25°C	0.4	0.4	0.2	0.1	0.2	0.2	0.2	0.2
ACTINOMYCETES									
Micropolyspora faeni	60°C	0.2	0.1	0.1	0.4	0.2	0.1	0.1	0.1
Thermoactinomyces vulgaris	60°C	0.2	0.2	0.1	0.2	0.2	0.2	0.1	0.1
" glaucus	37°C	0.4	0.4	0.4	0.2	0.2	0.2	0.4	0.4
White streptomyces (1)	37°C	0.4	0.4	0.4	0.2	0.4	0.4	0.4	0.4
" " (2)	37°C	0.4	0.1	0.4	0.4	0.4	0.2	0.4	0.4
" " (3)	25°C	0.4	0.1	0.4	0.2	0.4	0.2	0.6	0.4
Grey "	25°C	0.4	0.4	0.2	0.4	0.4	0.4	0.6	0.4
BACTERIA									
Bacillus spp (1)	60°C	0.2	0.08	0.1	0.02	0.08	0.06	0.06	0.06
" spp (2)	60°C	0.2	0.08	0.1	0.02	0.08	0.06	0.06	0.06
" licheniformis	37°C	0.4	0.1	0.2	0.04	0.2	0.08	0.1	0.08
" spp (3)	37°C	0.2	0.1	0.1	0.04	0.1	0.06	0.08	0.06
Lactobacillus spp (1)	37°C	1.0	0.6	0.4	0.4	0.8	0.4	0.4	0.4
Bacillus spp (4)	25°C	0.2	0.08	0.2	0.04	0.1	0.08	0.1	0.1
Lactobacillus spp (2)	25°C	1.4	0.8	1.0	0.4	1.0	0.8	0.8	0.8
Micrococcus spp	25°C	0.2	0.2	0.1	0.04	0.1	0.1	0.08	0.08
Pseudomonas spp	25°C	0.4	0.2	0.2	0.06	0.2	0.2	0.1	0.2

Table 16 - The minimum inhibitory concentrations of organic acids against micro-organisms occurring in hay, at p.H 6.0.

Acetic: sorbic = Acetic acid: sorbic acid 9:1 w/w
Propionic: sorbic = propionic acid: sorbic acid 9:1 w/w
n-butyric: sorbic = n-butyric acid: sorbic acid 9:1 w/w
propionic: n-butyric: sorbic = propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)

Micro-organism	Incubation temperature	Minimum inhibitory concentration %							
		Acetic acid	propionic acid	n-butyric acid	sorbic acid	Acetic: sorbic	propionic: sorbic	n-butyric: sorbic	propionic: n-butyric: sorbic
FUNGI									
Absidia ramosa	45°C	0.8	0.6	0.4	0.2	0.4	0.4	0.2	0.2
Aspergillus fumigatus	45°C	1.0	0.8	0.6	0.2	0.6	0.6	0.4	0.6
" glaucus spp	45°C	0.8	0.6	0.4	0.1	0.4	0.4	0.2	0.2
Mucor pusillus	45°C	0.8	0.4	0.4	0.2	0.4	0.2	0.2	0.2
Thermomyces lanuginosa	45°C	0.4	0.2	0.2	0.1	0.2	0.2	0.1	0.1
Aspergillus spp (1)	25°C	0.8	0.8	0.4	0.1	0.4	0.2	0.2	0.4
" spp (2)	25°C	0.8	0.8	0.4	0.1	0.2	0.2	0.2	0.2
" candidus	25°C	0.8	0.8	0.4	0.2	0.4	0.4	0.4	0.4
Mucor spp	25°C	0.6	0.4	0.4	0.2	0.2	0.2	0.2	0.2
Penicillium spp	25°C	0.8	0.8	0.4	0.2	0.4	0.4	0.2	0.4
ACTINOMYCETES									
Micropolyspora faeni	60°C	0.4	0.2	0.2	0.8	0.4	0.4	0.2	0.2
Thermoactinomyces vulgaris	60°C	0.4	0.4	0.2	0.6	0.4	0.4	0.2	0.2
" glaucus	37°C	0.6	0.8	0.8	0.6	0.6	0.6	0.8	0.8
White streptomyces (1)	37°C	0.8	0.6	0.8	0.6	0.6	0.6	0.8	0.8
" " (2)	37°C	0.8	0.2	0.8	0.8	0.8	0.4	0.6	0.4
" " (3)	25°C	0.8	0.4	0.6	0.6	0.8	0.4	0.6	0.6
Grey "	25°C	0.8	0.8	0.4	0.6	0.8	0.6	0.4	0.4
BACTERIA									
Bacillus spp (1)	60°C	0.4	0.2	0.2	0.08	0.2	0.2	0.2	0.2
" spp (2)	60°C	0.4	0.2	0.2	0.06	0.2	0.2	0.2	0.2
" licheniformis	37°C	1.0	0.4	0.4	0.1	0.6	0.4	0.4	0.4
" spp (3)	37°C	0.6	0.4	0.4	0.1	0.4	0.2	0.4	0.4
lactobacillus spp (1)	37°C	1.6	1.2	0.8	0.8	1.2	1.0	0.8	1.0
Bacillus spp (4)	25°C	0.6	0.2	0.6	0.1	0.4	0.2	0.4	0.4
lactobacillus spp (2)	25°C	1.8	1.4	1.4	1.0	1.4	1.4	1.4	1.4
Micrococcus spp	25°C	0.6	0.6	0.4	0.1	0.4	0.4	0.4	0.4
Pseudomonas spp	25°C	0.8	0.6	0.6	0.2	0.6	0.6	0.6	0.6

Table 17 - The minimum inhibitory concentrations of organic acids against micro-organisms occurring in hay at p.H 7.0.

Acetic: sorbic = Acetic acid: sorbic acid 9:1 w/w
Propionic: sorbic = propionic acid: sorbic acid 9:1 w/w
n-butyric: sorbic = n-butyric acid: sorbic acid 9:1 w/w
propionic: n-butyric: sorbic = propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)

Micro-organism	Incubation temperature	Minimum inhibitory concentration %							
		Acetic acid	propionic acid	n-butyric acid	sorbic acid	Acetic: sorbic	propionic: sorbic	n-butyric: sorbic	propionic: n-butyric: sorbic
FUNGI									
Absidia ramosa	45°C	1.4	1.0	0.8	0.4	0.8	0.6	0.6	0.6
Aspergillus fumigatus	45°C	1.6	1.2	0.8	0.4	1.0	0.8	0.6	0.6
" glaucus spp	45°C	1.2	1.0	0.8	0.2	0.8	0.6	0.6	0.6
Mucor pusillus	45°C	1.4	0.8	0.8	0.4	0.6	0.6	0.6	0.6
Thermomyces lanuginosa	45°C	0.6	0.4	0.4	0.2	0.4	0.4	0.2	0.2
Aspergillus spp (1)	25°C	1.4	1.2	0.8	0.2	0.8	0.6	0.4	0.6
Aspergillus spp (2)	25°C	1.4	1.2	0.8	0.2	0.6	0.8	0.6	0.8
Aspergillus candidus	25°C	1.4	1.4	0.8	0.4	0.8	1.0	0.8	1.0
Mucor spp	25°C	1.2	0.8	0.8	0.4	0.4	0.6	0.6	0.6
Penicillium spp	25°C	1.4	1.2	0.8	0.4	0.8	0.8	0.6	0.6
ACTINOMYCETES									
Micropolyspora faeni	60°C	0.6	0.4	0.4	1.4	0.6	0.8	0.6	0.6
Thermoactinomyces vulgaris	60°C	0.6	0.8	0.4	1.2	0.6	0.8	0.4	0.6
" glaucus	37°C	1.0	1.2	1.4	1.0	1.0	1.0	1.4	1.4
White streptomyces (1)	37°C	1.2	1.0	1.4	1.2	1.0	1.2	1.4	1.4
" " (2)	37°C	1.2	0.4	1.4	1.4	1.2	0.8	1.4	1.0
" " (3)	25°C	1.2	0.8	1.2	1.0	1.2	0.8	1.2	1.2
Grey "	25°C	1.4	1.2	0.8	1.2	1.2	1.2	1.0	1.0
BACTERIA									
Bacillus spp (1)	60°C	0.8	0.4	0.4	0.2	0.6	0.4	0.4	0.4
" " (2)	60°C	0.8	0.4	0.4	0.2	0.6	0.4	0.4	0.4
Bacillus licheniformis	37°C	1.6	0.8	0.8	0.4	1.2	0.6	0.8	0.8
Bacillus spp (3)	37°C	1.0	0.6	0.8	0.4	0.8	0.6	0.8	0.6
lactobacillus spp (1)	37°C	2.2	2.0	1.4	1.4	1.8	1.8	1.4	1.4
Bacillus spp (4)	25°C	1.0	0.6	1.2	0.4	0.8	0.6	1.0	1.0
lactobacillus spp (2)	25°C	2.4	2.2	2.0	1.8	2.2	2.2	2.0	2.0
Micrococcus spp	25°C	1.2	1.0	0.8	0.4	1.0	0.8	0.6	0.6
Pseudomonas spp	25°C	1.4	1.2	1.0	0.6	1.2	1.0	0.8	0.8

TABLE 18 - The minimum inhibitory concentrations of organic acids against micro-organisms occurring in hay at p.H 8.0.

Acetic: sorbic = Acetic acid: sorbic acid 9:1 w/w

Propionic: sorbic = propionic acid: sorbic acid 9:1 w/w

n-butyric: sorbic = n-butyric acid: sorbic acid 9:1 w/w

propionic: n-butyric: sorbic = propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)

At a p.H value of 5.0, the fungi were generally more resistant to these organic acids, than the actinomycetes and bacteria, whereas at p.H 8.0 these three groups of micro-organisms showed similar resistance. This may have been because fungi generally prefer slightly more acid conditions for growth, than the actinomycetes and bacteria and it was noticeable, throughout these experiments, that the fungi grew well on the controls at p.H 5.0 whereas most of the bacteria and all the actinomycetes tested grew weakly.

These results clearly demonstrate an increase in the anti-microbial activity of these organic acids, with a decrease in p.H, approximately ten to twenty times the inhibitory concentrations of these acids at p.H 5.0, being needed to prevent growth of the same micro-organisms at p.H 8.0.

Concentration of propionic acid	p.H of a 0.2M phosphate buffer
0.0%	7.0
0.1%	6.8
0.2%	6.6
0.4%	6.2
0.8%	5.1
1.6%	4.5

Table 19 - The effect, on the p.H of a 0.2M phosphate buffer, of the addition of various levels of propionic acid.

The effect of propionic acid on the p.H of the 0.2M phosphate buffer at p.H 7.0 used in these experiments, is shown in table 19. It can be seen that more than 0.4% propionic acid lowered the p.H by at least approximately one unit, and therefore the inhibitory activity of these organic acids may partly have been a p.H effect, where higher levels were used, especially when actinomycetes and bacteria were tested at lower p.H values. The apparently high resistance of Lactobacilli spp to these

organic acids may at least partly have been due to their ability to grow at low p.H values.

b) The activity of further preservatives against mixed spore suspensions

The screening of organic acids for their anti-microbial activity, at different p.H values, against a series of microbial pure cultures was considered to be a very detailed method for screening compounds as potential hay preservatives and it did not allow for the occurrence of less common micro-organisms which could have been more resistant to the preservatives than the micro-organisms tested.

To overcome these problems the technique using three sectioned petri-dishes was developed and it was used to screen several anti-microbial compounds against mixed spore suspensions from deteriorated hay.

The main disadvantage of this technique compared with the use of pure cultures, was that it demonstrated the concentrations of preservative which prevented the germination of microbial spores, but these probably would be less than the concentrations necessary to inhibit vegetative growth, as shown by Thornton (1963) for organic acids. Considering hay, which has been lying in the field for several days and is then baled with a high moisture level, it would contain actively growing micro-organisms and therefore higher levels of preservative, than those which prevent spore germination, would be necessary.

To examine possible differences in the results obtained by these two methods, the first three-sectioned petri dish experiment was designed to determine the minimum inhibitory levels of propionic acid for the mixed population of hay, in order to make a comparison with the results for pure cultures shown in Tables 15 - 18. The results are shown in Table 20, and they suggest that slightly lower levels of propionic acid were able to prevent spore germination than could inhibit mycelial growth, but as with the results in Tables 15 - 18, there appeared to be little difference between the resistance of the actinomycetes and the fungi and also an increase in p.H caused a decrease in the anti-microbial activity of propionic acid.

Compound	Minimum inhibitory concentration.%					p.H
	Actinomycetes incubated at			Fungi incubated at		
	60°C	37°C	25°C	45°C	25°C	
Propionic acid	0.06	0.06	0.06	0.1	0.1	5.0
	0.1	0.1	0.1	0.2	0.2	6.0
	0.2	0.2	0.2	0.4	0.6	7.0
	0.4	0.6	0.6	0.6	0.8	8.0

Table 20 - The minimum inhibitory concentrations of propionic acid against spores from deteriorated hay.

Further experiments were then carried out testing other compounds and the minimum inhibitory levels of these compounds are summarised in Table 21.

Formaldehyde, paraformaldehyde and glutaraldehyde are all well known anti-microbial agents. Methyl and propyl hydroxybenzoates are commonly used as preservatives in the pharmaceutical industry and because the activity of propyl hydroxybenzoate was enhanced by a lowering of p.H, it was considered possible that a propionic acid; propyl hydroxybenzoate mixture would have acted synergistically and therefore this mixture was tested in these experiments and compared with a propionic acid; sorbic acid mixture (9:1 w/w). Sodium nitrite is a well known preservative used in the food industry notably as a meat preservative. Butylamine has been used to preserve vegetables and propylene glycol was included because it was added to the commercial hay preservative 'Hay Shield' marketed by Feed Service Ltd., as a wetting agent, in order to encourage the spreading of the preservative after application to the hay.

Sodium and methyl propionates had been tested for their antimicrobial activity during work on media that were selective for actinomycetes, as has been described earlier in this work. Their inhibitory levels were found to be considerably higher than for propionic acid and they were therefore not included in these experiments.

Compound	Minimum inhibitory concentration %					p.H
	Actinomycetes incubated at			Fungi incubated at		
	60°C	37°C	25°C	45°C	25°C	
Formaldehyde	0.002	0.02	0.02	0.02	0.04	5.0
	0.002	0.02	0.02	0.02	0.04	6.0
	0.004	0.02	0.02	0.02	0.02	7.0
	0.008	0.04	0.04	0.01	0.02	8.0
Para-formaldehyde	0.002	0.02	0.02	0.06	0.08	5.0
	0.004	0.04	0.06	0.08	0.1	6.0
	0.006	0.06	0.06	0.1	0.1	7.0
	0.01	0.06	0.08	0.1	0.1	8.0
Glutaral-dehyde	0.06	0.2	0.1	0.1	0.2	5.0
	0.04	0.1	0.08	0.08	0.2	6.0
	0.02	0.06	0.04	0.06	0.1	7.0
	0.01	0.02	0.02	0.04	0.08	8.0
methyl hydroxy-benzoate	0.06	0.1	0.1	0.06	0.08	5.0
	0.1	0.2	0.2	0.1	0.1	6.0
	0.08	0.1	0.2	0.1	0.1	7.0
	0.1	0.2	0.2	0.1	0.1	8.0
propyl hydroxy-benzoate	0.008	0.01	0.01	0.008	0.008	5.0
	0.02	0.04	0.04	0.01	0.02	6.0
	0.04	0.06	0.04	0.02	0.02	7.0
	0.04	0.06	0.04	0.01	0.02	8.0
propionic acid:sorbic acid(90:10 w/w)	0.06	0.08	0.08	0.06	0.08	5.0
	0.1	0.1	0.2	0.1	0.2	6.0
	0.2	0.2	0.2	0.2	0.2	7.0
	0.6	0.6	0.6	0.4	0.4	8.0
propionic acid: propyl hydroxy benzoate (90:10 w/w)	0.06	0.06	0.08	0.06	0.06	5.0
	0.1	0.1	0.2	0.1	0.1	6.0
	0.2	0.2	0.2	0.2	0.2	7.0
	0.4	0.4	0.4	0.2	0.4	8.0
sodium nitrite	0.02	0.06	0.1	0.2	0.4	5.0
	0.6	1.0	1.0	2.0	2.0	6.0
	1.0	1.4	1.6	2.6	2.8	7.0
	1.2	1.8	1.8	3.0	3.2	8.0
secondary butylamine	0.8	1.0	1.0	0.6	1.2	5.0
	0.8	1.0	1.0	0.6	1.0	6.0
	0.6	0.8	0.8	0.4	0.8	7.0
	0.4	0.6	0.6	0.2	0.6	8.0
propylene glycol	2.6	2.6	2.8	3.2	3.6	5.0
	2.8	3.0	3.0	3.4	3.6	6.0
	2.0	3.6	3.8	3.6	3.6	7.0
	3.2	3.8	4.0	3.6	3.4	8.0

Table 21 - The minimum inhibitory concentrations of various anti-microbial compounds against spores from deteriorated hay.

Formaldehyde and paraformaldehyde demonstrated a greater activity against the micro-organisms tested, than propionic acid, and this activity was little affected by changes in p.H. Their activity against the thermophilic actinomycetes was particularly strong, possibly because they became more active at higher temperatures, and the paraformaldehyde probably depolymerised to formaldehyde more rapidly. Glutaraldehyde showed similar inhibitory levels to formaldehyde at p.H 8.0 but a lowering of the p.H. decreased its activity, until at p.H 5.0 it was less inhibitory than propionic acid. Propyl hydroxybenzoate was more antimicrobially active than both methyl hydroxybenzoate and propionic acid and it demonstrated similar inhibitory levels to formaldehyde at p.H values of 6, 7 and 8, however a lowering of the p.H to 5.0 enhanced its activity further especially against the actinomycetes, although this would possibly have been a p.H effect. Propyl hydroxybenzoate appeared to have been slightly more active against the fungi than the actinomycetes. At p.H 8.0 the propionic acid; propyl hydroxybenzoate mixture was slightly more inhibitory than the propionic acid - sorbic acid mixture, probably because the propyl hydroxybenzoate was more active at p.H 8.0 than the sorbic acid. The sorbic acid did not appear to have enhanced the activity of propionic acid which contrasted with the earlier results shown in Tables 15 to 18, where the addition of sorbic acid increased the inhibitory activity of propionic acid. At p.H values of 5, 6 and 7 there appeared to be little difference between the mixtures, propionic acid with sorbic acid and propionic acid with propyl hydroxybenzoate.

Sodium nitrite demonstrated similar inhibitory levels to propionic acid against the actinomycetes, at p.H 5.0, but it was less active against the fungi. However, an increase in p.H caused its activity to decrease considerably more than in the case of propionic acid therefore

at p.H 8.0 it had a lower anti-microbial activity. Secondary butylamine demonstrated a low anti-microbial activity at p.H 5.0 against both fungi and actinomycetes, however, its activity increased slightly with a rise in p.H, although this could have been a p.H effect due to this compound being alkaline. Propylene glycol demonstrated low activity at all the p.H values tested.

Considering these results for assessing the potential of these compounds as hay preservatives, formaldehyde, paraformaldehyde, glutaraldehyde and propyl hydroxybenzoate had the best possibilities. They were therefore examined, along with the organic acids, as preservatives for hay stored in dewar flasks, where propyl hydroxybenzoate created application problems because it was a solid, insoluble in water, however, it was applied to the hay dissolved in propionic acid. Methyl hydroxybenzoate, sodium nitrite, secondary butylamine and propylene glycol were considered to have little potential as hay preservatives.

C) The use of hay stored in 4.5 litre dewar flasks

The chemicals, which in the previous experiments demonstrated inhibitory activity at low levels, against hay micro-organisms were examined for their ability to prevent re-wetted fresh hay from deteriorating when stored in 4.5 litre dewar flasks. In addition the effect of the hay moisture level, and the freshness of the hay on the quantities of preservative necessary for control, were studied.

In dewar flask experiments 1 to 5, the volatile fatty acids were extracted from the hay by soaking in 0.6N H_2SO_4 for seven days, which probably gave recoveries of between 60% and 90% (Table 12). In experiments 6 - 10 the volatile fatty acids were extracted by heating the hay in kilner jars which gave over 97% recovery (Table 12). In all these experiments sorbic acid was extracted by soaking the hay in 0.6N H_2SO_4 for seven days.

In all these experiments glucosamine was estimated using the colourimetric method of Tsuji et al (1969).

i) Experiment one

Because only a limited number of dewar flasks were available, replication of treatments in these experiments was impractical, therefore the initial experiment was set up to determine the flask to flask variation, in similarly treated hay, of the parameters to be studied. This was done by filling six flasks with hay from the same bale, which had been re-wetted to 41% moisture, the hay in three of these flasks being treated with 0.5% propionic acid.

Various measurements were recorded from the hay during and after storage, as previously described, and the results are summarised in table 22, the temperature recordings being shown in fig 25.

These results show that similar hay in dewar flasks had different heating patterns and contained different microbial numbers after storage. This demonstrates that the results obtained by the use of these experiments can only be used as a guideline for the comparative effectiveness of various treatments as hay preservatives.

ii) Experiment two and three

In the second experiment the commercial hay preservative ' Hay Shield' marketed by Feed Services Ltd., which contained approximately 30% propionic acid along with a few other ingredients, was compared with the most effective anti-microbial organic acid mixture as tested in agar media, which was the propionic acid: n-butyric acid: sorbic acid mixture (45:45:10 w/w/w). Sorbic acid was added to Hay Shield at the rate of 4% (w/w) to examine the possibility of such an addition improving the preservative action of Hay Shield and finally the organic acid mixture was mixed with a 40% solution of formaldehyde in water in the ratio 7:3 (v/v) acid: formaldehyde, to determine whether the two compounds would act synergistically.

In the third experiment the same treatments as in the second experiment were employed, except that different application rates were used and the Hay Shield treatement was omitted because sorbic acid had enhanced its activity in the second experiment.

The results for the second experiment are shown in table 23 and

Treatment	Application Rate %	Moisture level %		propionic acid level %		Heating max temp °C	Heating degree days above 20°C	glucosamine level of hay %	Diamino-pimelic-acid level %	Microbial spore counts X 10^4/g hay: Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	Microbial counts X 106/g hay: Aerobic bacteria incubated at 60°C	37°C	25°C	Microbial counts X 10^2/g hay: lactobacilli 37°C	25°C	Anaerobes 37°C	25°C	Colifo 37°C
		Initial	Final	Initial	Final																	
Initial Hay	-	-	-	-	-	-	-	0.53	0.08	<0.1	39	102	15	84	<0.1	1500	2000	11	15	<0.1	<0.1	<0.1
Untreated	-	37	48	-	-	37	110	2.41	0.27	119	210	225	84	210	7.2	6000	12000	18	43	<0.1	8	7
Untreated	-	38	51	-	-	28	18	1.02	0.14	27	127	135	99	168	0.4	5000	8000	12	37	<0.1	2	2
Untreated	-	48	47	-	-	24	7	0.97	0.12	8	139	165	100	150	<0.1	7000	6000	11	29	<0.1	5	<0.1
Propionic acid	0.5	39	46	0.26	<0.01	30	44	1.25	0.09	102	167	166	74	136	<0.1	3700	7000	12	19	<0.1	11	13
Propionic acid	0.5	42	52	0.11	<0.01	36	107	2.69	0.19	177	350	269	109	242	18	2700	4000	49	88	<0.1	10	2
Propionic acid	0.5	43	49	0.33	<0.01	36	119	3.82	0.27	98	184	210	85	204	<7.8	4000	6000	22	131	<0.1	9	13

Table 22 - Results for the first dewar flask experiment - before and after 28 days storage

< = Less than

	glucosamine level of hay %	Diamino-pimelic-acid level %	Microbial spore counts X 10^4/g hay					Microbial counts X 106/g hay			Microbial counts X 10^2/g hay						p.H	Reference for statistical Analysis
			Actinomycetes incubated at			Fungi incubated at		Aerobic bacteria incubated at			lactobacilli		Anaerobes		Coliforms			
			60°C	37°C	25°C	45°C	25°C	60°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C		
	0.53	0.08	<0.1	39	102	15	84	<0.1	1500	2000	11	15	<0.1	<0.1	<0.1	<0.1	5.7	-
10	2.41	0.27	119	210	225	84	210	7.2	6000	12000	18	43	<0.1	8	7	15	6.8	A
8	1.02	0.14	27	127	135	99	168	0.4	5000	8000	12	37	<0.1	2	2	13	7.2	B
7	0.97	0.12	8	139	165	100	150	<0.1	7000	6000	11	29	<0.1	5	<0.1	24	7.1	C
4	1.25	0.09	102	167	166	74	136	<0.1	3700	7000	12	19	<0.1	11	13	37	7.5	D
7	2.69	0.19	177	350	269	109	242	18	2700	4000	49	88	<0.1	10	2	4	7.7	E
9	3.82	0.27	98	184	210	85	204	<7.8	4000	6000	22	131	<0.1	9	13	9	7.4	F

Results for the first dewar flask experiment - before and after 28 days storage

s than

statistical analysis (P = <.05)

Actinomycetes incubated at 60°C
B + C <A,D,E,F.

" " at 37°C
B + C < A
E > ABCDF

" " at 25°C
A > BCD
F > B
E > ABCDF

Fungi incubated at 45°C No difference

" " " 25°C B E > BCD
A F > C.D.

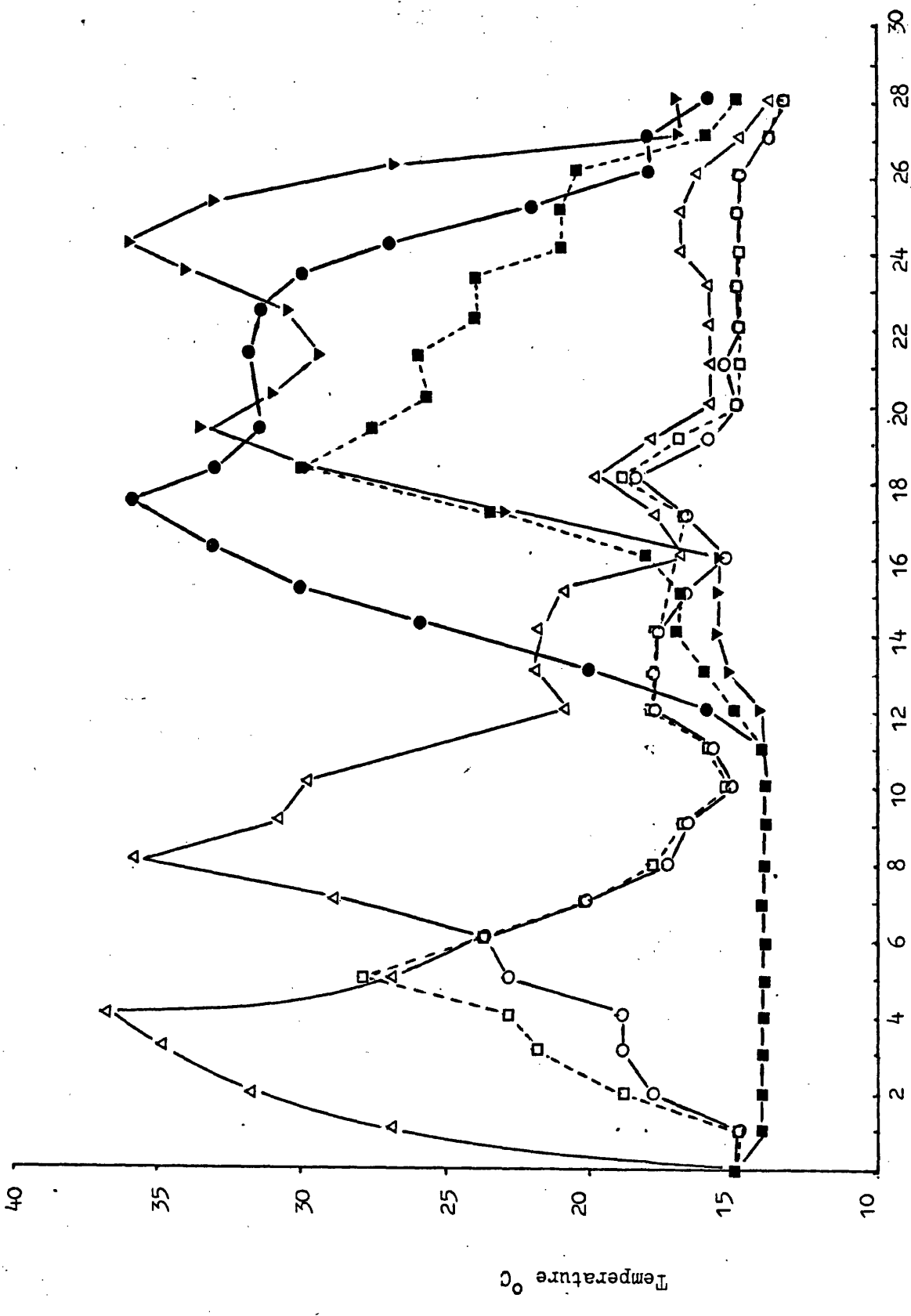


Fig 25 Temperature-time graph for 1st dewar flask experiment (△) (□) (○) Untreated hay (●) (▼) (■) 0.5% propionic acid treated hay. (40% moisture hay)

the temperature recordings in fig 26. The results for the third experiment are shown in table 24 and the temperature recordings in fig 27.

In these experiments Hay Shield was not as effective as the organic acid mixture probably because there was less acid present, although the results from experiment two suggested that the addition of sorbic acid to Hay Shield at a concentration of 4%, increased the effectiveness of Hay Shield. Formaldehyde appeared to have been inactivated in some way, after its application to hay, because its addition to the organic acids reduced their effectiveness, probably due to dilution.

iii) Experiments four, five, six and seven

In these experiments a few organic acid mixtures were tested for their ability to preserve hay at various initial moisture contents, in order to, firstly compare treatments and secondly, to obtain a relationship between the minimum effective organic acid level and the initial moisture content of the hay.

The results for these experiments are shown in tables 25, 26, 27, and 28 and the temperature recordings in figs 28, 29, 30 and 31.

Experiments four and five suggested there was little difference between the propionic: n-butyric: sorbic acids mixture, the propionic acid: sorbic acid mixture and propionic acid alone, in the prevention of either heating or an increase in microbial spore numbers in the hay.

In experiment six, the addition of sorbic acid appears to have

Treatment	Application rate %	Initial organic acid level			Final organic acid level			Moisture level %		Heating max temp °C	Heating degree days above 20°C	Glucosamine level of hay %	Diaminopimelic acid levels of hay %	Microbial spore counts X 10^4/g hay.						Microbial counts x 10^0/g hay Aerobic bacteria incubated at			Microbial counts x 10^2/g hay						p.H	Reference for statistical analysis
		propionic	n-butyric	sorbic	propionic	n-butyric	sorbic	Initial	Final					Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C		60°C	37°C	25°C	lactobacilli 37°C	lactobacilli 25°C	Anaerobes 37°C	Anaerobes 25°C	Coliforms 37°C	Coliforms 25°C		
Initial Hay	-	-	-	-	-	-	-	-	-	-	-	0.39	-	<0.1	10	30	12	14		0.6	22	56	3	7	<0.1	<0.1	<0.1	<0.1	6.5	-
Untreated	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.001	28	41	39	30	1.28	-	98	65	128	212	330		36	78	200	872	*	<0.1	1	5	18	7.8	A
Hay Shield	0.5	0.05	-	-	<0.01	<0.01	<0.001	29	34	25	11	0.74	-	<0.1	129	213	103	350		<0.1	35	47	1	*	<0.1	<0.1	2	13	7.2	B
Hay Shield: sorbic acid 96: 4 w/w	0.5	0.05	-	0.006	<0.01	-	<0.001	31	29	21	1	0.86	-	<0.1	10	16	69	330		<0.1	1	14	<0.1	1	<0.1	<0.1	<0.1	2	6.8	C
propionic acid: n-butyric acid: sorbic acid: 45:45:10 w/w/w (mixture A)	0.5	0.10	0.09	0.02	0.02	0.03	0.002	30	24	22	4	0.64	-	<0.1	4	9	95	172		<0.1	0.4	0.6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	6.9	D
mixture A+ 40% formaldehyde in water (70:30 v/v)	0.5	0.05	0.06	0.01	<0.01	<0.01	<0.001	28	26	20	0	1.60	-	<0.1	1	1	26	95		<0.1	0.4	0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	7.0	E

Table 23 - Results for the second dewar flask experiment, before and after 22 days storage

* Plates overgrown by fungi.

< = Less than

Statistical analysis ($P < .05$)

Actinomycetes incubated at 60°C A > B,C,D,E.
" " " 37°C A + B > C,D,E.
" " " 25°C A,B, > C,D,E.
Fungi " " 45°C A > B,C,D,E.
" " " 25°C A,B,C > D,E.

Fig 26

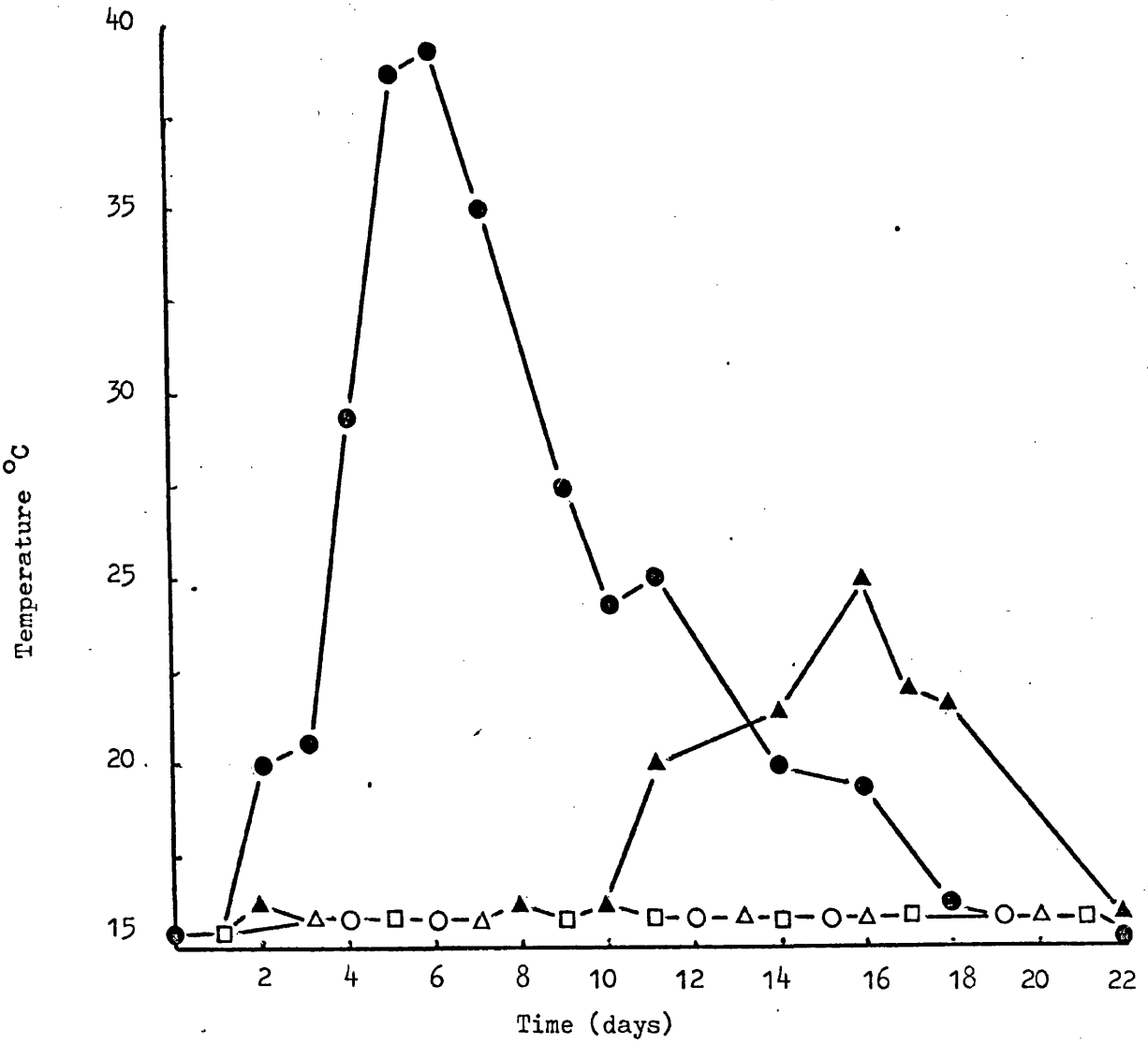


Fig 26 - temperature - time graph for the second dewar flask experiment (●) untreated (▲) 0.5% Hay Shield (□) 0.5% Hay Shield: sorbic acid mixture (O) 0.5% organic acid mixture (△) 0.5% organic acid: formaldehyde mixture (30% moisture hay).

Treatment	Application Rate %	Moisture level % Initial	Moisture level % Final	Initial organic acid level propionic	Initial organic acid level n-butyric	Initial organic acid level sorbic	Final organic acid level propionic	Final organic acid level n-butyric	Final organic acid level sorbic	Heating max temp °C	Heating degree days above 20°C	glucosamine level of hay %	Diamino-pimelic acid level of hay %	Microbial spore counts X 10^4/g hay Actinomycetes incubated at 60°C	Actinomycetes incubated at 37°C	Actinomycetes incubated at 25°C	Fungi incubated at 45°C	Fungi incubated at 25°C	Microbial counts X 10^0/g hay aerobic bacteria incubated at 60°C	aerobic bacteria incubated at 37°C	aerobic bacteria incubated at 25°C	Microbial counts X 10^2/g hay lactobacilli 37°C	lactobacilli 25°C	Anaerobes 37°C	Anaerobes 25°C	Coliforms 37°C	Coliforms 25°C	p.H	Reference for statistical Analysis
Initial Hay	-	-	-	-	-	-	-	-	-	-	-	1.1	0.07	0.6	0.2	0.4	1.2	1.2	<0.1	29	240	7	5	<0.1	<0.1	<0.1	<0.1	7.0	-
Untreated	-	34	42	-	-	-	-	-	-	43	85	3.2	0.18	0.4	2000	70	72	304	10	900	200	127	532	49	83	17	17	7.7	A
Hay shield:sorbic acid 96:4 w/w	0.25	32	27	0.05	-	0.008	<0.01	-	<0.001	34	81	3.1	0.14	0.3	158	87	123	258	1.5	240	50	204	738	13	42	41	38	8.1	B
propionic acid: n-butyric acid: sorbic acid. 45:45:10 (w/w/w) (mixture A)	0.25	29	28	0.12	0.11	0.05	<0.01	<0.01	<0.001	16.5	0	2.1	0.22	0.2	76	52	64	247	1.5	35	4	40	104	<0.1	<0.1	<0.1	<0.1	7.5	C
Mixture A	0.5	33	28	0.27	0.24	0.12	0.03	0.05	0.007	17.5	0	1.7	0.13	0.3	24	20	51	223	5	15	3	71	28	<0.1	<0.1	<0.1	<0.1	7.1	D
Mixture A + 40% formaldehyde in water 70: 30 (v/v) (mixture B)	0.25	34	28	0.12	0.13	0.04	<0.01	<0.01	<0.001	27.5	29	1.4	0.16	0.2	16	21	53	154	3.5	20	4	81	204	<0.1	<0.1	<0.1	12	7.3	E
Mixture B	0.5	30	29	0.23	0.20	0.07	0.04	0.04	0.008	19.5	0	2.1	0.15	0.15	17	15	31	73	3.5	25	4	41	41	<0.1	<0.1	<0.1	<0.1	7.7	F

Table 24 - Results for the third dewar flask experiment - before and after 22 days storage

< = Less than

statistical analysis ($P < .05$)

Actinomycetes incubated at 60°C - No differences
" " " 37°C - A + B >, D,E,F.
" " " 25°C - No differences
Fungi " " 45°C - No differences
" " " 25°C - No differences

Fig 27

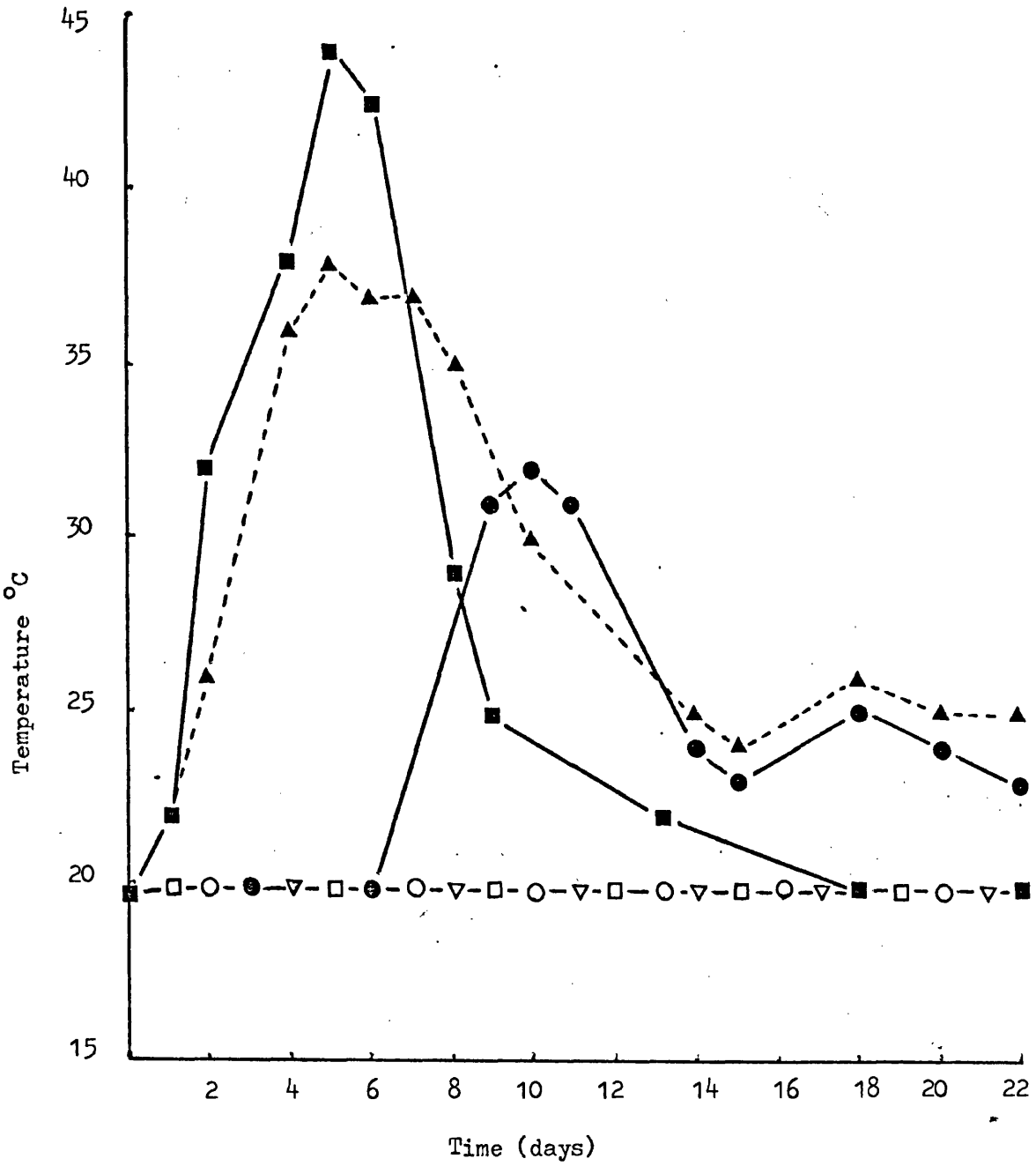


Fig 27 - Temperature- time graph for the third dewar flask experiment (■) Untreated (▲) 0.25% Hay Shield: sorbic acid mixture (□) 0.25% organic acid mixture (O) 0.5% organic acid mixture (●) 0.25% organic acids: formaldehyde mixture (▽) 0.5% organic acids: formaldehyde mixture (32% moisture hay)

improved the preservative action of propionic acid whereas with experiment seven propionic acid alone delayed heating for longer than the propionic acid; sorbic acid mixture.

iv) Experiment eight

This experiment was designed to compare the preservative activity of glutaraldehyde with both propionic acid and a propionic acid: sorbic acid mixture (9.1 w/w). Because the inhibitory activity of glutaraldehyde was stronger at higher p.H values in agar media (Table 21), it was not used in combination with organic acids, as was formaldehyde, in experiments two and three.

The results are shown in table 29 and the temperature recordings in fig 32a and 32b.

Glutaraldehyde appeared to be ineffective as a hay preservative probably because, as with formaldehyde, it reacted chemically with components of the hay which rendered it inactive. The 1.0% treatment with propionic acid; sorbic acid mixture slightly reduced heating compared with the 1.0% propionic acid treatment.

v) Experiment nine

In dewar flask experiment nine, a solution of propyl hydroxybenzoate in propionic acid (10:90 w/w), a solution of sorbic acid in propionic acid (10:90 w/w), paraformaldehyde and a paraformaldehyde: sodium nitrite mixture (70 : 30 w/w) were all compared as hay preservatives.

The results are shown in table 30 and the temperature recordings

Treatment	Application Rate %	Moisture level % Initial	Moisture level % Final	Initial organic acid levels propionic	Initial organic acid levels n-butyric	Initial organic acid levels sorbic	Final organic acid levels propionic	Final organic acid levels n-butyric	Final organic acid levels sorbic	Heating max temp °C	Heating degree days above 20°C	glucosamine level of hay %	Diamino-pimelic acid level %	Microbial spore counts X 10^4/g hay Actinomycetes incubated at 60°C	Actinomycetes incubated at 37°C	Actinomycetes incubated at 25°C	Fungi incubated at 45°C	Fungi incubated at 25°C	Microbial counts x 10^6/g hay Aerobic bacteria incubated at 60°C	Aerobic bacteria incubated at 37°C	Aerobic bacteria incubated at 25°C	Microbial counts x 10^2/g hay lactobacilli 37°C	lactobacilli 25°C	Anaerobes 37°C	Anaerobes 25°C	Coliforms 37°C	Coliforms 25°C	p.H	Reference for statistical analysis
Initial Hay	-	-	-	-	-	-	-	-	-	-	-	0.6	0.07	1	5	30	4	80	<0.1	7	4	17	39	<0.1	7	<0.1	<0.1	5.8	-
Untreated	-	42	62	-	-	-	-	-	-	60	132	2.4	0.21	30	407	313	515	423	4	70	33	328	502	19	32	78	181	7.3	A
propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)	0.5	44	66	0.23	0.24	0.10	<0.01	<0.01	<0.001	44	132	3.0	0.22	20	132	67	218	129	2	30	28	72	117	8	21	41	123	7.8	B
propionic acid: sorbic acid 9:1 (w/w)	0.5	45	58	0.38	-	0.05	<0.01	-	<0.001	34	99	9.6	0.24	47	604	621	197	381	1.3	32	104	89	609	<0.1	<0.1	59	77	7.4	C
propionic acid	0.5	42	58	0.41	-	-	<0.01	-	-	53	125	2.6	0.17	34	429	392	208	407	4	67	41	57	515	7	8	102	84	7.4	D

Table 25 - Results for the fourth dewar flask experiment - before and after 24 days storage

< = Less than

Statistical analysis ($P < .05$)

Actinomycetes incubated at 60°C - No differences
" " " 37°C - No differences
" " " 25°C - No differences
Fungi " " 45°C - No differences
" " " 25°C - A > C,D.

Treatment	Application Rate %	Moisture level %		Initial organic acid levels %			Final organic acid levels %			Heating max degree temp °C	Heating days above 20°C	Glucosamine level of hay %	Diamino-pimelic acid level of hay %
		Initial	Final	propionic	n-butyric	sorbic	propionic	n-butyric	sorbic				
Initial hay	-	-	-	-	-	-	-	-	-	-	-	0.5	0.04
Untreated	-	38	52	-	-	-	-	-	-	33	56	2.4	0.12
propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)·mixture A	0.5	42	49	0.18	0.19	0.03	<0.01	<0.01	<0.001	29	64	2.7	0.18
Mixture A	0.75	43	39	0.24	0.26	0.06	<0.01	<0.01	<0.001	42	200	3.8	0.12
propionic acid: sorbic acid: 9:1 w/w mixture B	0.5	43	39	0.41	-	0.05	<0.01	-	<0.001	57	141	3.2	0.25
Mixture B	0.75	40	43	0.63	-	0.11	<0.01	-	<0.001	37	147	1.9	0.28
propionic acid	0.5	43	42	0.38	-	-	<0.01	-	-	42	286	2.7	0.05
Propionic acid	0.75	38	40	0.72	-	-	<0.01	-	-	40	138	2.0	0.20

Treatment	Microbial spore counts X 10^4/g hay					Microbial counts X 10^6/g hay			Microbial counts X 10^2/g hay						p.H	Reference for statistical Analysis
	Actinomycetes incubated at			Fungi incubated at		Aerobic bacteria incubated at			lactobacilli		Anaerobes		Coliforms			
	60°C	37°C	25°C	45°C	25°C	60°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C		
Initial hay	<0.1	1	7	2	4	<0.1	4	3	29	81	<0.1	13	2	11	6.1	-
Untreated	*	103	182	107	115	*	3200	9600	117	712	29	102	53	111	7.3	A
propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)·mixture A	*	84	175	102	121	*	2400	4000	304	342	44	117	71	85	7.8	B
Mixture A	*	117	119	93	106	*	6200	1700	612	172	85	131	19	41	7.9	C
propionic acid: sorbic acid: 9:1 w/w mixture B	*	73	132	84	115	*	4000	3100	518	623	23	110	35	43	8.1	D
Mixture B	*	152	137	99	141	*	7600	5400	529	689	27	147	42	57	7.4	E
propionic acid	*	97	215	91	122	*	4000	2500	428	413	33	81	36	19	7.7	F
Propionic acid	*	153	194	81	137	*	3600	1100	319	272	42	75	16	18	7.3	G

Table 26 - Results for the fifth dewar flask experiment - before and after 36 days storage

Statistical analysis ($P < .05$)

No significant differences

* Plates overgrown by a spreading Bacillus spp

< = Less than

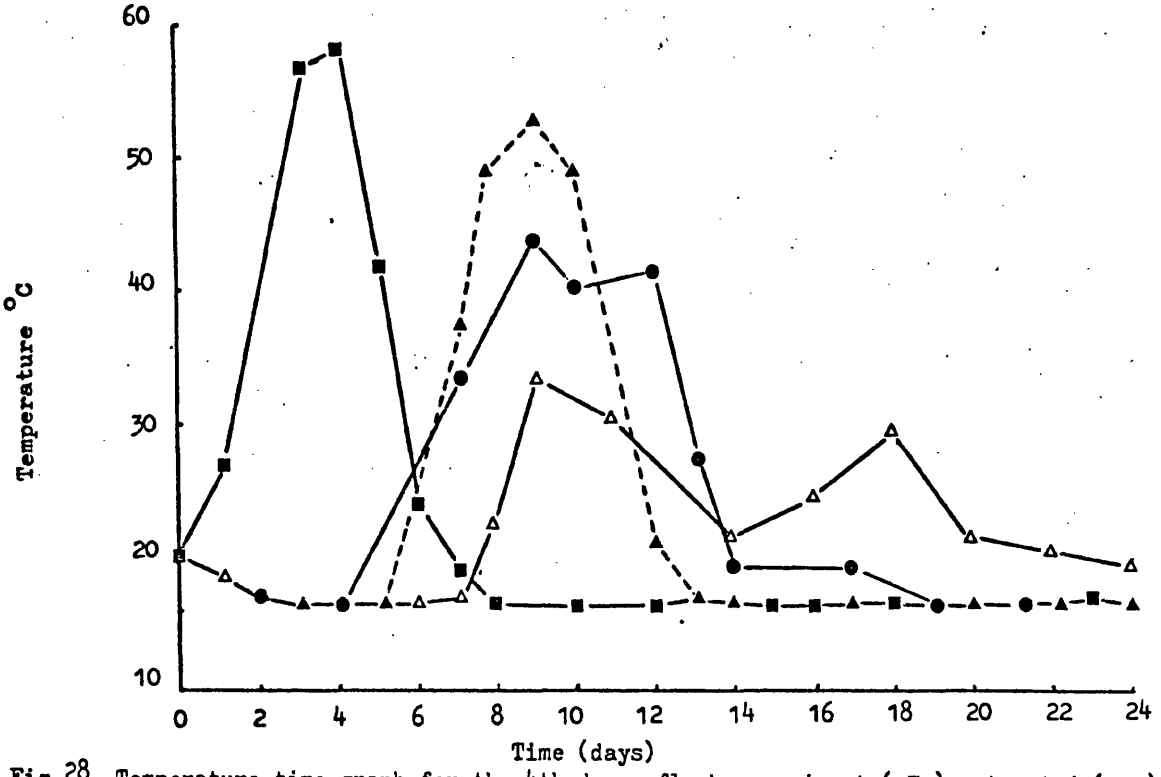


Fig 28 Temperature-time graph for the 4th dewar flask experiment (■) untreated (●) 0.5% organic acid mixture (△) 0.5% propionic & sorbic acids (▲) 0.5% propionic acid.

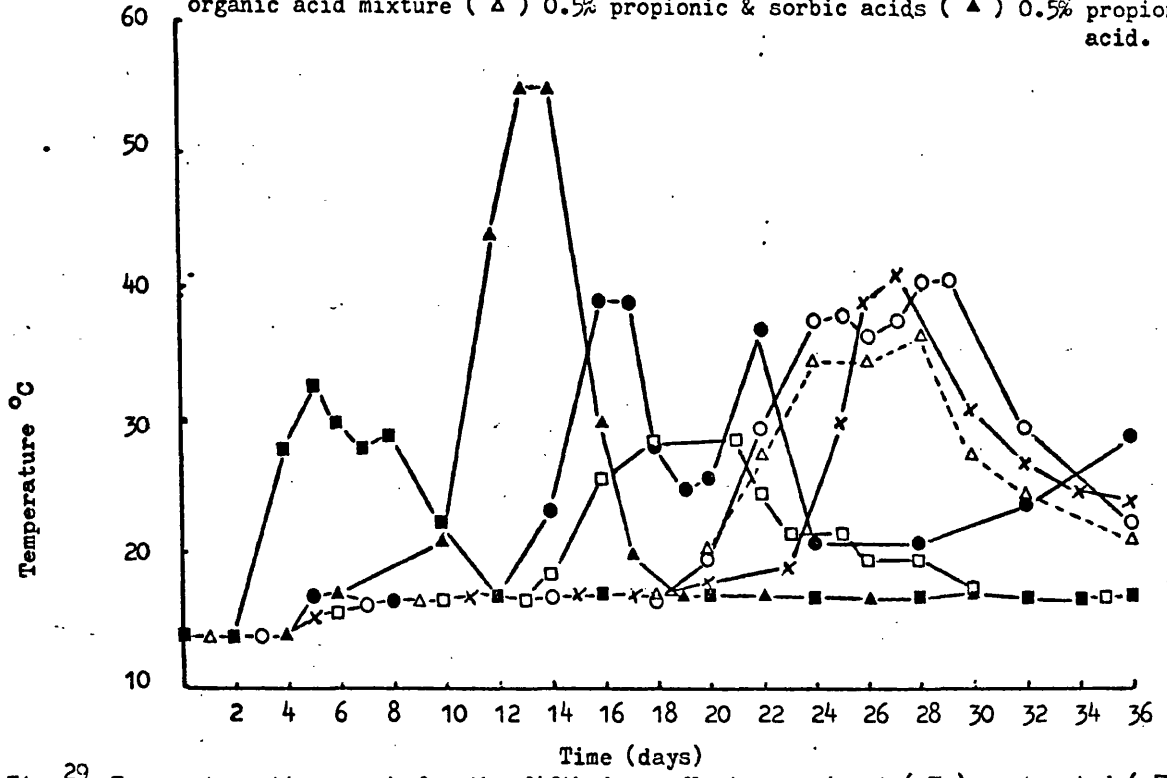


Fig 29 Temperature-time graph for the fifth dewar flask experiment (■) untreated (□) 0.5% organic acids (○) 0.75% organic acids (▲) 0.5% propionic: sorbic acids (△) 0.75% propionic : sorbic acids (●) 0.5% propionic acid (✕) 0.75% propionic acid. (40% moisture hay)

Treatment	Application Rate %	Moisture level % Initial	Moisture level % Final	Initial organic acid level % propionic	Initial organic acid level % sorbic	Final organic acid level % propionic	Final organic acid level % sorbic	Heating max temp °C	Heating degree days above 20°C	Glucosamine level of hay %	Diamino-pimelic acid level of hay %	Microbial spore counts X 10^4/g hay: Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	Microbial counts X 10^6/g hay: Aerobic bacteria incubated at 60°C	37°C	25°C	p.H	Reference for statistical analysis
Initial Hay	-	-	-	-	-	-	-	-	-	1.00	0.03	6.4	7.3	6.7	9	6.4	<0.1	16	120	5.8	-
Untreated	-	34	33	-	-	-	-	50	287	2.75	0.18	289	590	531	8	89	*	3.6	8	7.8	A
propionic acid	0.5	35	31	0.28	-	0.01	-	56	317	3.05	0.35	84	314	408	20	88	*	200	14	8.4	B
propionic acid: sorbic acid 9:1 w/w(mixture A)	0.5	32	34	0.40	0.032	<0.01	0.002	53	177	1.70	0.24	33	464	344	213	492	*	23	24	8.2	C
												65	503	420	299	273	*	8	24	7.3	D
propionic acid	0.75	34	35	0.72	-	<0.01	-	48	225	2.28	0.27	11	144	286	34	353	*	12	50	7.4	E
mixture A	0.75	33	33	0.62	0.078	<0.01	0.008	22	4	3.07	0.23										

Statistical analysis ($P < .05$)

Actinomycetes incubated at 60 C
A > B,C,D,E.

Actinomycetes incubated at 37°C
A,C,D,> E.

Actinomycetes incubated at 25°C
No differences

Fungi incubated at 45°C - No differences

" " " 25°C - No differences

Table 27 - Results for the sixth dewar flask experiment - before and after 66 days storage

* Plates overgrown by a spreading Bacillus spp

< = Less than

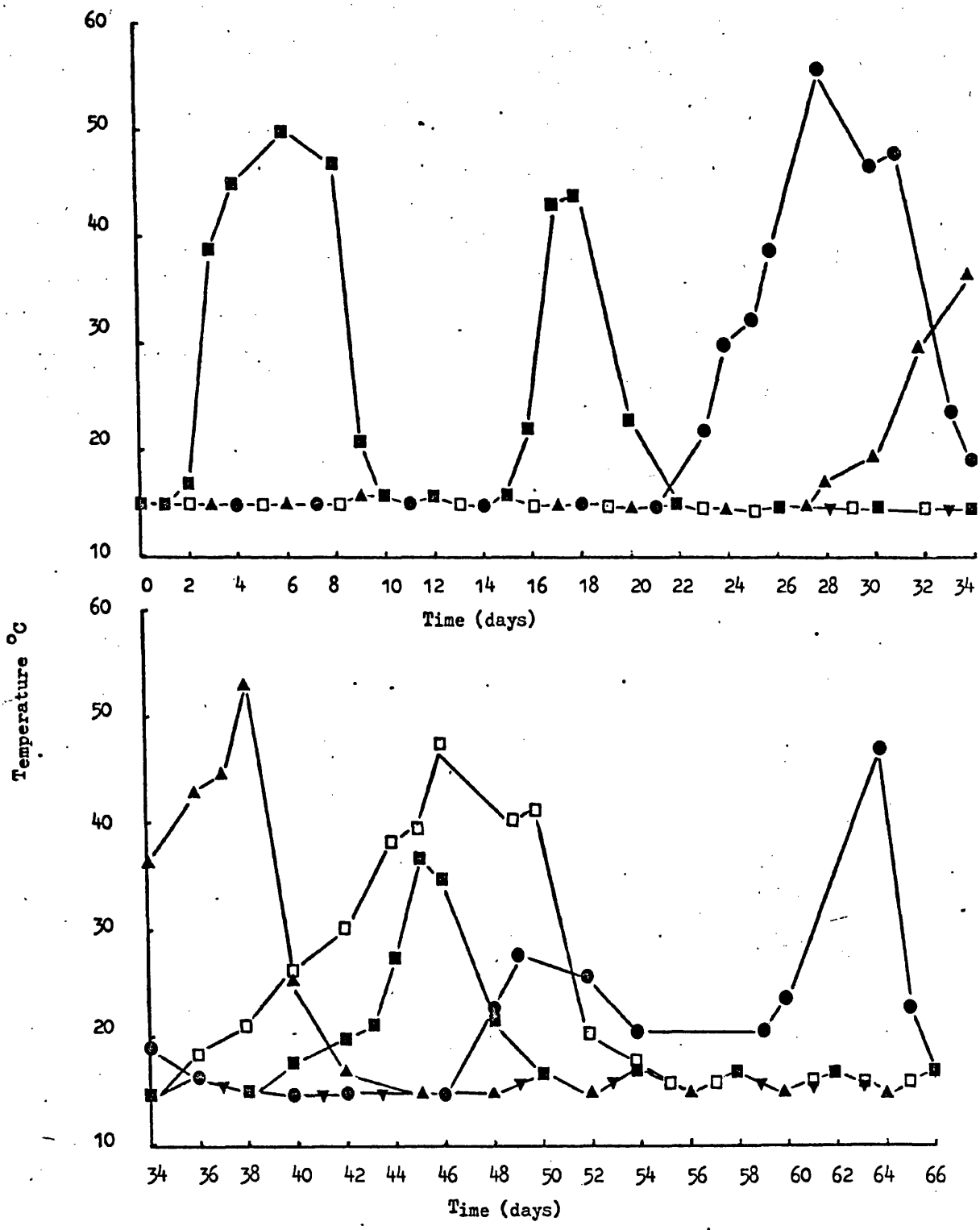


Fig 30 Temperature-time graph for the sixth dewar flask experiment (■) untreated (●) 0.5% propionic acid (□) 0.75% propionic acid (▲) 0.5% propionic: sorbic acids (▼) 0.75% propionic: sorbic acids. (34% moisture hay)

Treatment	Applications rate %	Moisture level %		Initial organic acid level %		Final organic acid level %		Heating max temp °C	Heating degree days above 20°C	Glucosamine level of hay %	Diamino-pimelic acid level of hay %	Microbial spore counts X 10^4/g hay					Microbial counts X 10^6/g hay			p.H	Reference for statistical Analysis
		Initial	Final	propionic	sorbic	propionic	sorbic					Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	aerobic bacteria incubated at 60°C	37°C	25°C		
Initial hay	-	-	-	-	-	-	-	-	-	1.08	0.03	6.4	7.3	6.7	9	6.4	<0.1	16	120	5.8	-
Untreated	-	44	30	-	-	-	-	65	697	2.50	0.53	149	420	485	36	600	*	1900	23	8.2	A
propionic acid	1.0	44	30	1.02	-	<0.01	-	34	226	3.27	0.59	44	203	188	411	633	*	2100	2500	7.9	B
propionic acid: sorbic acid (9:1 w/w) Mixture A	1.0	46	31	0.68	0.086	<0.01	0.002	35	212	2.72	0.24	29	281	304	127	608	*	4700	350	7.7	C
propionic acid	1.5	47	34	1.30	-	0.21	-	16	0	1.47	0.28	31	239	330	17	357	*	1.7	2.1	7.4	D
mixture A	1.5	47	36	1.40	0.120	0.034	0.033	22	5	2.87	0.58	9	172	302	37	591	*	3.0	12	7.3	E

Table 28 - Results for the seventh dewar flask experiment - before and after 66 days storage

* plates overgrown by a spreading Bacillus spp

< = Less than

Statistical analysis ($P < .05$)

Actinomycetes incubated at	60°C	A > B,C,D,E.
"	37°C	No differences
"	25°C	A > B,C,D,E.
		A,C,D,E E.
Fungi	45°C	B > A,C,D,E.
"	25°C	A,B,C,E > D.

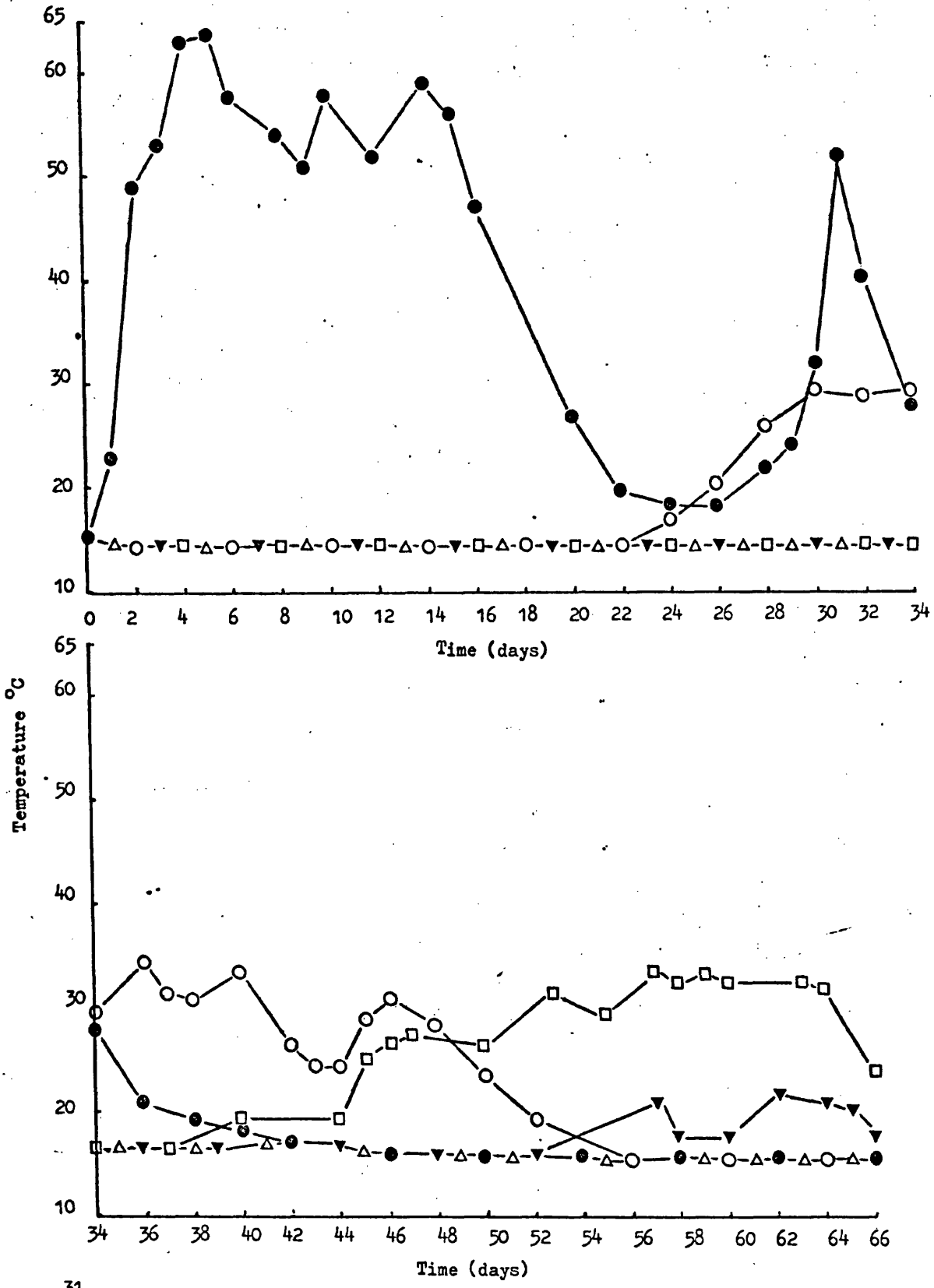


Fig 31 Temperature-time graph for the seventh dewar flask experiment (●) untreated (□) 1.0% propionic acid (△) 1.5% propionic acid (○) 1.0% propionic: sorbic acids (▼) 1.5% propionic: sorbic acids. (45% moisture hay)

in fig 33.

The 1.5% treatment with the paraformaldehyde: sodium nitrite mixture reduced heating when compared with all the treatments except the 1.5% propionic acid: sorbic acid mixture and the 1.5% propionic acid: propyl hydroxybenzoate mixture, treatements, although in the case of the latter two preservatives mixtures, it appears the hay was beginning to heat at the termination of the experiment.

vi) Notes on experiments 1 - 9

The actinomycete and fungal spore counts, which are important when considering the danger to health aspect of deteriorated hay, were statistically analysed and significant differences ($P < .05$) are shown with the relevant table of results. Bacterial counts were not analysed because a high proportion of the agar plates contained actinomycete species which produced anti-bacterial compounds, thus the developement of bacterial colonies was inhibited. Therefore the general aerobic bacterial counts should be viewed with caution.

The counts of anaerobic bacteria, Lactobacilli and Coliforms were discontinued after the fifth experiment for two reasons. Firstly, their numbers were too low, when compared with the general aerobic bacterial count, for them to be considered to play an important role in the deterioration of hay. Secondly, they did not appear to have been more able to resist the preservative action of the organic acids, as was originally considered possible.

Because of the difficulties in estimating microbial numbers in hay, in these experiments, it was considered that the complete control of

Treatment	Application Rate %	Moisture level % Initial	Moisture level % Final	propionic acid level % Initial	propionic acid level % Final	Heating max temp °C	Heating degree days above 20°C	glucosamine level of hay %	Diamino-pimelic-acid level %	Microbial spore counts X 10^4/g hay: Actinomycetes incubated at 60°C	Actinomycetes incubated at 37°C	Actinomycetes incubated at 25°C	Fungi incubated at 45°C	Fungi incubated at 25°C
Initial Hay	-	-	-	-	-	-	-	0.53	0.08	<0.1	39	102	15	84
Untreated	-	37	48	-	-	37	110	2.41	0.27	119	210	225	84	210
Untreated	-	38	51	-	-	28	18	1.02	0.14	27	127	135	99	168
Untreated	-	48	47	-	-	24	7	0.97	0.12	8	139	165	100	150
Propionic acid	0.5	39	46	0.26	<0.01	30	44	1.25	0.09	102	167	166	74	136
Propionic acid	0.5	42	52	0.11	<0.01	36	107	2.69	0.19	177	350	269	109	242
Propionic acid	0.5	43	49	0.33	<0.01	36	119	3.82	0.27	98	184	210	85	204

Microbial counts X 106/g hay: Aerobic bacteria incubated at 60°C	Aerobic bacteria incubated at 37°C	Aerobic bacteria incubated at 25°C	Microbial counts X 10^2/g hay: lactobacilli 37°C	lactobacilli 25°C	Anaerobes 37°C	Anaerobes 25°C	Coliforms 37°C	Coliforms 25°C	p.H	Reference for statistical Analysis
<0.1	1500	2000	11	15	<0.1	<0.1	<0.1	<0.1	5.7	-
7.2	6000	12000	18	43	<0.1	8	7	15	6.8	A
0.4	5000	8000	12	37	<0.1	2	2	13	7.2	B
<0.1	7000	6000	11	29	<0.1	5	<0.1	24	7.1	C
<0.1	3700	7000	12	19	<0.1	11	13	37	7.5	D
18	2700	4000	49	88	<0.1	10	2	4	7.7	E
<7.8	4000	6000	22	131	<0.1	9	13	9	7.4	F

Table 22 - Results for the first dewar flask experiment - before and after 28 days storage

< = Less than

statistical analysis (P = <.05)

Actinomycetes incubated at 60°C
B + C < A, D, E, F.

" " at 37°C
B + C < A
E > ABCDF

" " at 25°C
A > BCD
F > B
E > ABCDF

Fungi incubated at 45°C No difference

" " " 25°C B E > BCD
A F > C.D.

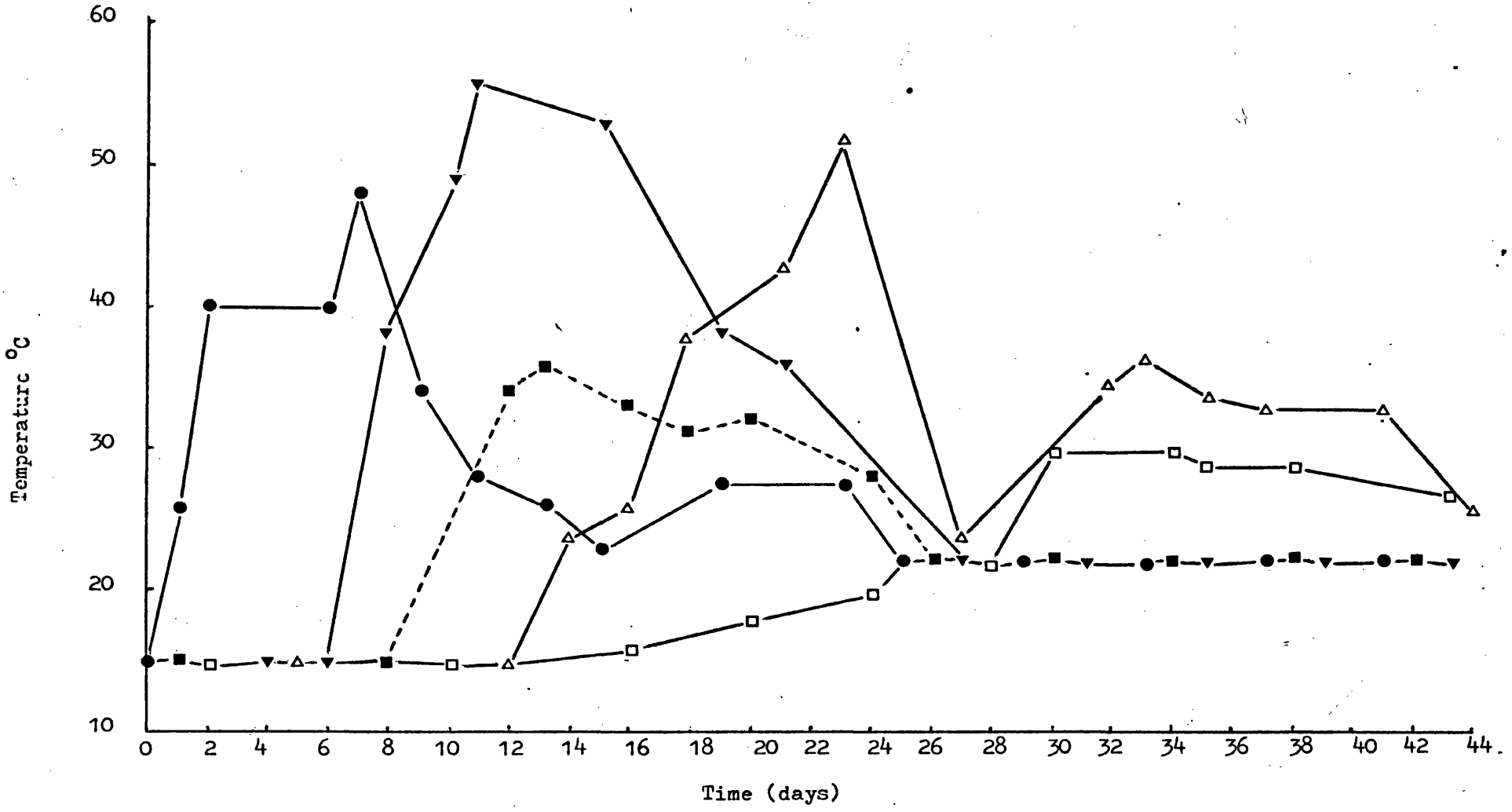


Fig 32a Temperature-time graph for the eighth dewar flask experiment (●) untreated (■) 0.75% propionic acid (△) 1.0% propionic acid (▼) 0.75% propionic : sorbic acids (□) 1.0% propionic: sorbic acids. (32% moisture hay)

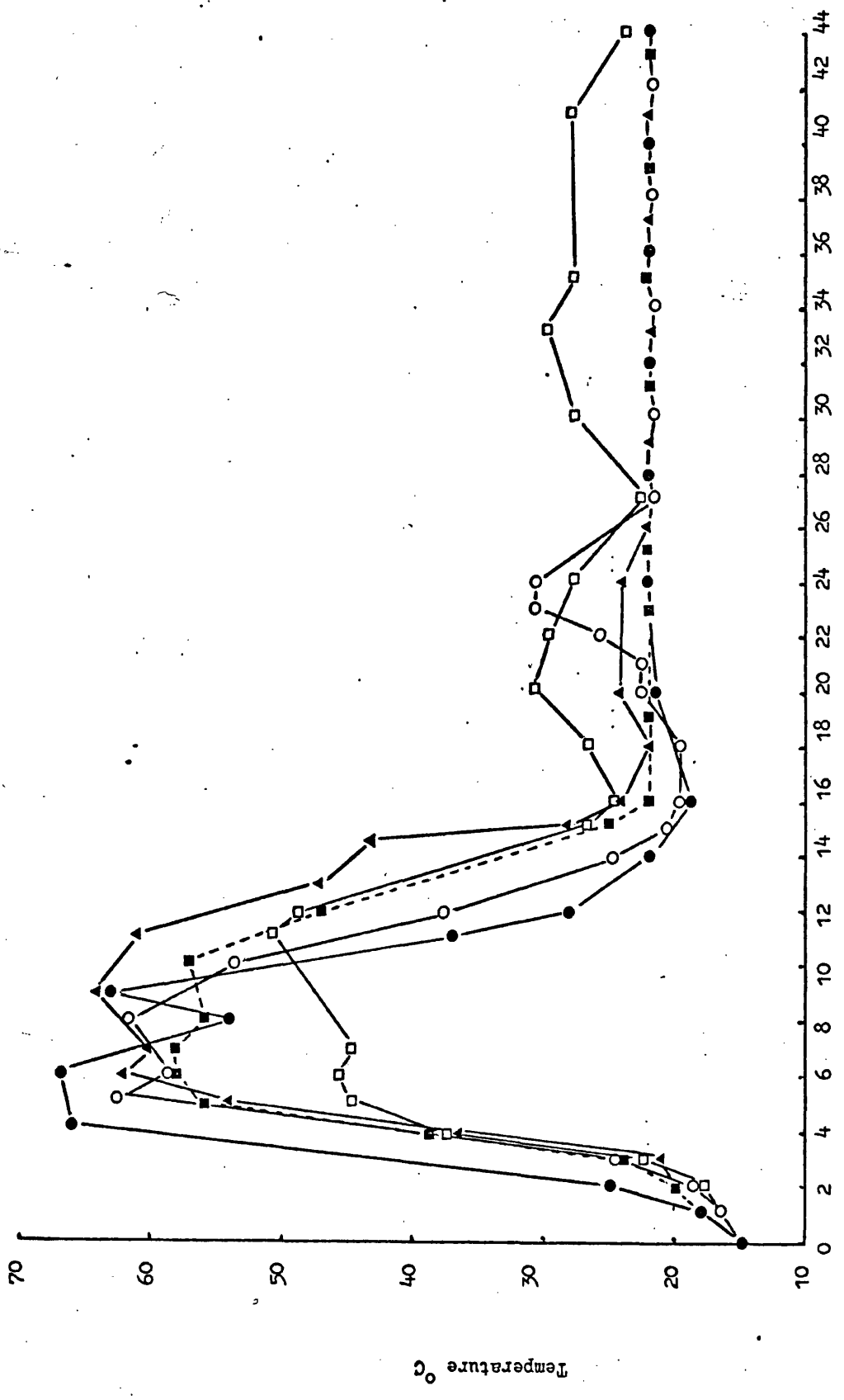


Fig 32b Temperature-time graph for the eighth dewar flask experiment (●) 0.5% Glutaraldehyde (Gl)(□) 0.75% Gl (○) 1.0% Gl (■) 1.25% Gl (▲) 1.5% Gl. (32% moisture hay)

Treatment	Application Rate %	Moisture level % Initial	Moisture level % Final	Initial organic acid level % propionic	Initial organic acid level % sorbic	Final organic acid level % propionic	Final organic acid level % sorbic	Heating max temp oC	Heating degree days above 20°C	Glucosamine level of hay %	Diamino-pimelic acid level of hay %
Initial hay	-	-	-	-	-	-	-	-	-	0.49	0.05
Untreated	-	40	45	-	-	-	-	46	273F	2.97	0.31
propionic acid: sorbic acid 9:1(w/w)	1.0	40	48	1.07	0.102	< 0.01	0.003	33	159F	1.82	0.27
propionic acid: sorbic acid w/w 9:1	1.5	41	35	1.14	0.145	<0.01	0.003	32	52 NF	1.73	0.22
propionic acid: propyl hydroxybenzoate 9:1 (w/w)	1.0	39	36	0.76	-	<0.01	-	57	598NF	3.51	0.56
propionic acid :propyl hydroxy-benzoate 9:1 w/w	1.5	40	38	1.03	-	<0.01	-	35	57 NF	1.80	0.21
paraformaldehyde	1.0	40	32	-	-	-	-	64	513F	3.38	0.46
paraformaldehyde	1.5	41	47	-	-	-	-	52	480F	3.61	0.44
paraformaldehyde: Na NO_2 7:3 (w/w)	1.0	40	32	-	-	-	-	57	457F	2.77	0.61
paraformaldehyde $NaNO_2$ 7:3 (w/w)	1.5	41	51	-	-	-	-	38	169F	1.82	0.33
Untreated	-	43	29	-	-	-	-	61	571F	3.19	0.49

Table 30 - Results for the ninth dewar flask experiment - before and after 40

F = Heating appeared to have finished
NF = Heating appeared not to have finished

< = Less than

Microbial spore counts x 10^4/g hay					Microbial counts x 10^6/g hay			p.H	Reference for statistical analysis
Actinomycetes incubated at			Fungi incubated at		Aerobic bacteria incubated at				
60°C	37°C	25°C	45°C	25°C	60°C	37°C	25°C		
12	42	50	32	84	<0.1	150	450	6.2	-
310	268	207	11	153	1.9	3800	4500	7.8	A
225	204	167	9	600	0.8	3500	3600	7.4	B
97	454	217	6	238	<0.1	140	230	7.3	C
415	483	542	102	136	52	70	130	8.2	D
161	88	238	48	221	4.0	100	112	7.4	E
453	392	384	426	321	113	800	2000	8.3	F
381	562	401	73	77	51	1040	4000	8.4	G
277	208	421	329	359	125	1400	4200	8.6	H
153	127	287	84	465	17	1400	1600	7.6	I
404	434	445	434	454	84	6400	2800	7.7	J

days storage.

Statistical analysis (P< .05)

Actinomycetes incubated at 60°C - No differences

" " " 37°C C,D,G,J,> B
G >A
c > E,H,I.
D > E,H,I.
F.G.J. E.
G > FHI
J >H.I.

" " " 25°C No differences

Fungi " " 45°C H> A,B,C,D,E,G,I.
F + J >A,B,C,D,E,
G,H,I.

D > C

" " " 25°C B,I,J, >A
B >C,D,E,F,G.
I,J > D
H.I.J.>G.

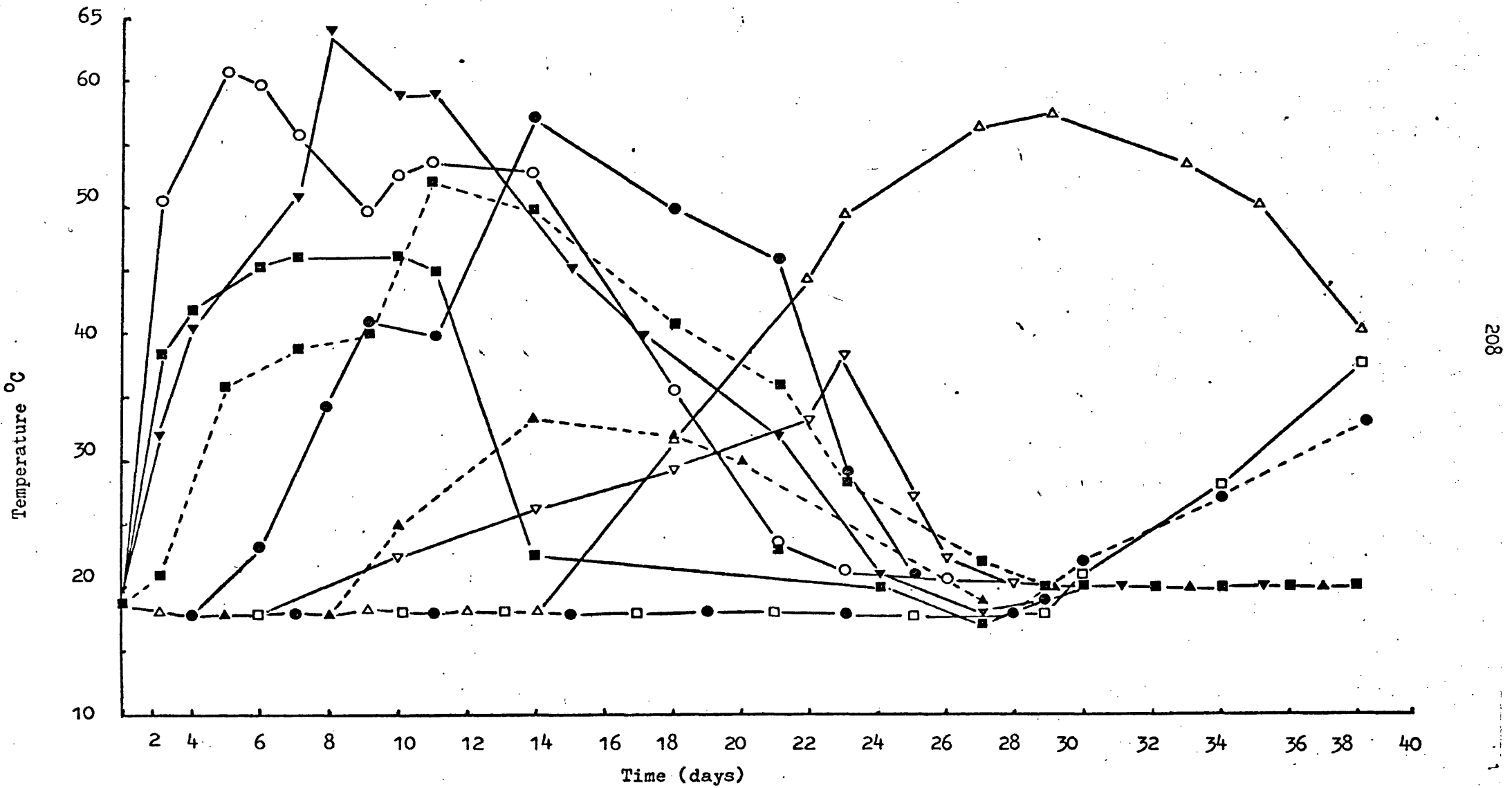


Fig 33 Temperature-time graph for the ninth dewar flask experiment (—■—) (—○—) untreated (--▲--) 1.0% propionic: sorbic acids (--●--) 1.5% propionic: sorbic acids (—△—) 1.0% propionic acid: propyl hydroxybenzoate (PH) (—□—) 1.5% P.H. (—▼—) 1.0% Paraformaldehyde (PF) (--■--) 1.5% P.F. (—●—) 1.0% P.F. + $NaNO_2$ (—▽—) 1.5% P.F. + $NaNO_2$. (40% moisture hay).

heating of the hay was the most accurate means of determining, whether or not, a certain treatment had prevented microbial deterioration of that hay.

v) General observations of experiments 1 - 9

Organic acid treatments always delayed the heating of treated hays, possibly because either they extended the lag phase of the growth of the storage micro-organisms or acid resistant micro-organisms, which were initially present in the hay as a small proportion of the total microflora, gradually increased in number and degraded the acids until the levels were reduced sufficiently to permit the growth of the remaining micro-organisms. A third possibility was that the organic acids slowly reacted with components in the hay until their levels were sub-inhibitory.

The first and third of these possibilities appears unlikely because when organic acid levels were determined on treated hay before and after storage, the organic acids almost totally disappeared when heating had taken place. Whereas, when no heating had occurred, their levels decreased but the acids were usually present after storage at significantly higher levels than with heated hays.

The addition of organic acids frequently reduced the maximum temperature attained by the hay, but the quantity of heat produced, expressed as degree days, was not necessarily decreased. This suggested that the total microbial activity, expressed as heat produced, was not reduced by the treatments, but that the rate of activity was slowed down.

The results shown in fig 34 summarise the minimum organic acid levels which completely prevented the heating of hay at various moisture levels, in dewar flasks, and the maximum levels which permitted heating. For these results all the organic acids examined and the various organic acid mixtures were considered to have the same preservative activity, and in experiments two and three the quantity of propionic acid in the Hay Shield was calculated as were the acid levels in the formaldehyde mixtures, the formaldehyde being considered unimportant.

Fig 34 also shows that different types of hay with the same moisture contents, required different levels of organic acid for preservation. For example, in experiment three, the 0.25% organic acid mixture treatment prevented heating of 32% moisture hay, whereas in experiment eight, 32% moisture hay heated when treated with either 1.0% propionic acid or 1.0% of the propionic acid; sorbic acid mixture. A further example was shown in experiment seven, where heating was prevented in 46% moisture hay, by treatment with 1.5% propionic acid however, in experiment 9, 40% moisture hay treated either with 1.5% of the propionic acid: sorbic acid mixture or with 1.5% of the propionic acid, propyl hydroxybenzoate mixture, heated considerably. In experiments eight and nine, a fresh, greener hay was used, suggesting that for a given initial moisture level, the greener the hay the more preservative would be needed to prevent deterioration.

Various compounds and chemical mixtures were tested in these dewar flask experiments and there was no substantial evidence to suggest that any of these treatments had been more effective than propionic acid in preventing hay from deteriorating.

In general terms, a relationship between the actinomycete spore

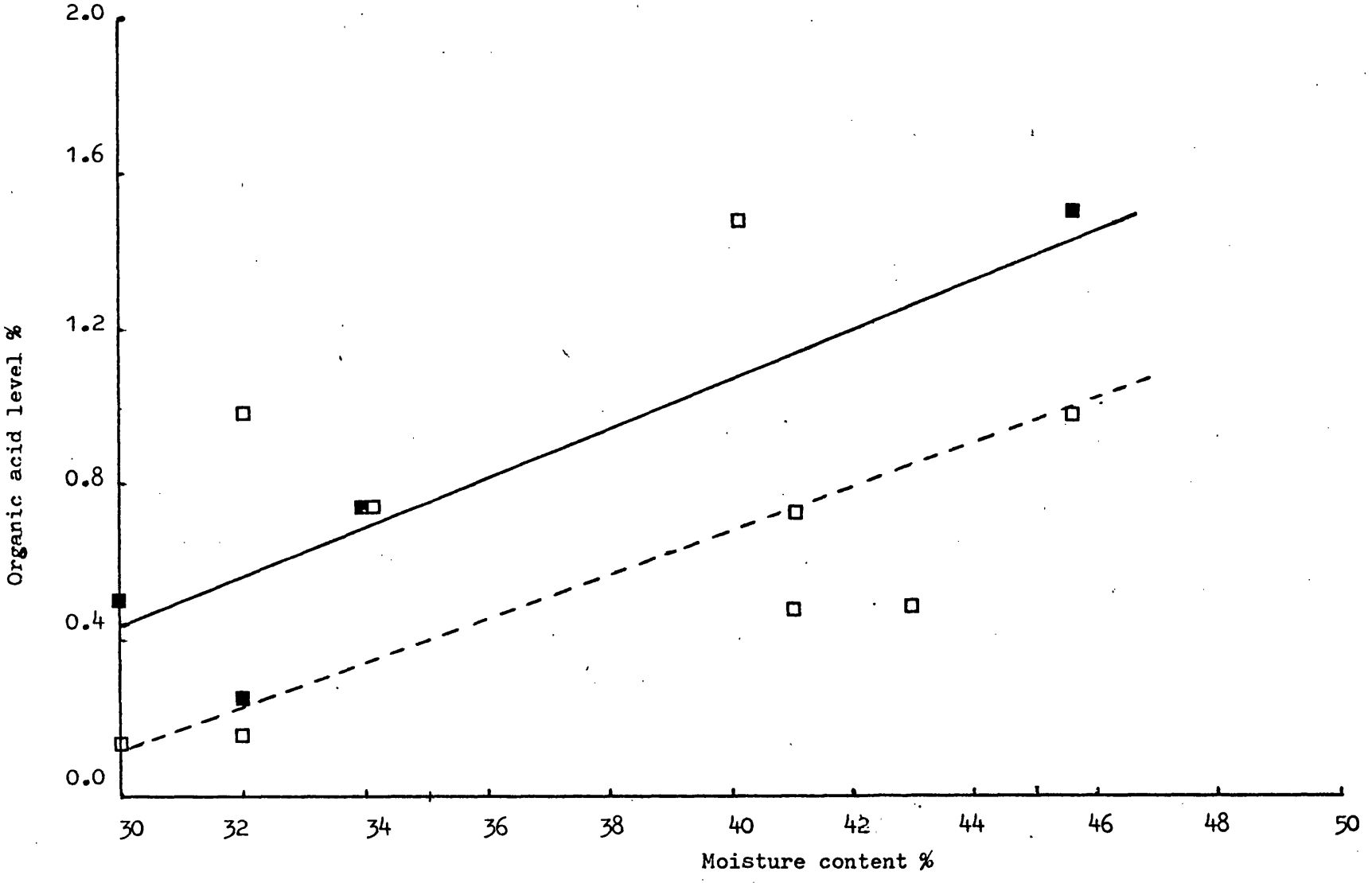


Fig 34 The effect of various levels of organic acids applied to hay in dewar flask experiment 1 - 9 and the relationship with the moisture content of the hay. (—■—) Minimum level which totally inhibited heating, (--□--) highest level which permitted heating of the hay.

count and the fungal spore counts, existed with the amount of heating that occurred in the hay, although the final spore counts did not usually show the differences in numbers that the variations in heating suggested would have occurred.

This relationship was not always observed, and there were some experiments where the final spore counts did not correlate with the heating of the hay. For example, in experiment three (Table 24) the untreated hay heated to 43°C and the organic acid mixture treated hay did not heat, however, no significant differences between the thermophilic actinomycete spore counts and the thermophilic fungal spore counts for the two hays were found. In experiment six (Table 27), the untreated hay, the 0.5% propionic acid treated hay and the 0.5% propionic acid: sorbic acid mixture treated hay, heated to over 50°C, whereas the 0.75% propionic acid; sorbic acid mixture treated hay did not heat and yet the thermophilic fungal spore counts showed no significant differences between treatments.

The final thermophilic actinomycete spore numbers were found to be reduced by chemical treatments to a greater extent than the other micro-organisms studied. This was possibly because the treatments had reduced the maximum temperature the hay had attained, which in turn had reduced the growth of these particular micro-organisms more than the majority of the microflora because they required higher temperatures for growth.

Considerable variation was observed between the final microbial counts of hay stored in dewar flasks which had similar initial moisture levels and had heated to similar extents. If, for example experiments

four and five are compared, in both cases the initial moisture levels were approximately 40% and the amount of heating was often similar, however, the final actinomycete and fungal spore counts were considerably higher in the hay from experiment four. Why these variations should have occurred is not clear but it was probably because different types of hay were used in the two experiments and these would have contained different nutrient levels, which would have affected, not only the nutrients available to the micro-organisms but also the water activities of the hays, and therefore, different growth conditions would have occurred, giving rise to different microflora. It is of interest to note the estimated bacterial counts were lower in experiment four than five, although this could have been due to antibiotics being produced by the larger number of actinomycetes present in the agar plates from experiment four.

In experiments three, six and seven, hay stored in dewar flasks which had shown little or no heating, had higher microbial counts, notably mesophilic micro-organisms, after storage than when it had originally been packed into the dewar flasks. Only in some of the treated hays in dewar flask experiment two, had the microbial counts not increased during storage. These results suggest that the organic acids, at these levels, on hay were not microbicidal but microbistatic, or nearly so, and that micro-organisms were slowly growing or at least sporulating, but not rapidly enough to cause significant heating of the hay. The fungal spores which germinated at 45°C increased in number in some hays which had not heated (Table 35), although with these hays the dominant fungi incubated at this temperature were the thermotolerant Aspergillus spp and Absidia spp rather than the true thermophiles including Thermomyces lanuginosa, Penicillium dupontii, Mucor pusillus

and Malbranchea pulchella var sulfurea, which were dominant on heated hays.

Double heating peaks occasionally occurred, for example in the untreated and 0.5% propionic acid treated hay in the sixth dewar flask experiment (fig 30). These could possibly have been due to a microbial succession occurring in the dewar flask hay, similar to that which was described for baled hay earlier in this report, and could be explained by the developement of an initial microflora utilising the readily available nutrients and that their activity caused the first temperature rise. As these nutrients were exhausted there occurred a decrease in microbial activity therefore the temperature dropped, however, the activity of micro-organisms capable of degrading larger molecules would then increase and as molecules, including cellulose and hemicellulose, were degraded, these micro-organisms and secondary invaders in utilising the new source of nutrients, would cause the temperature to rise again.

Hays which had heated, generally had higher p.H values (7.3 - 8.5) than unheated hays (p.H 6.5 - 7.3). However, measured p.H values for hay were inconsistent in that they could not be related to the amount of heating which had taken place in the hay. This was possibly due to the uneven distribution of nutrients and water that occurs within hay and which would in turn would cause variations in the microbial growth resulting in an uneven distribution of microbial metabolic products throughout the hay.

Estimations of the percentage dry weight of microbial material in hay by glucosamine and diaminopimelic acid determinations, gave disappointing results because although a relationship was observed between these calculated microbial constituent levels and the quantity

of heating which had occurred in the hay expressed as degree days, the differences in these levels between good hays and deteriorated hays were small when compared with the differences in estimated microbial numbers. In addition no real connection was evident either between fungal spore counts and glucosamine levels, or between actinomycete spore counts and bacterial counts and diaminopimelic acid levels.

vi) Experiment ten

The final dewar flask experiment, was designed to study the sequence of changes, if any, that occurred when damp hay treated with 1.0% of the propionic acid: sorbic acid mixture (90: 10 w/w) deteriorated while stored in dewar flasks.

It was hoped that information from this experiment would shed light on some of the problems highlighted by the first nine experiments, for example why the organic acid levels decreased on stored hay and why some hays showed double heating peaks.

In addition to the various measurements made in experiments 1 - 9, in this dewar experiment a few additional parameters were examined. Firstly, cellulolytic bacteria, actinomycetes and fungi were examined to see at what stage in the deterioration process their numbers increased. Secondly, the presence of fungal and actinomycete spores, which could germinate and grow in strictly anaerobic conditions, was looked for, because it was considered the oxygen tension in dewar flasks probably became very low when the hay deteriorated. Thirdly, the possible developement of propionic acid resistant micro-organisms was studied and the propionic acid and sorbic acid levels on the hay were followed during storage, in order firstly to determine any relationship between

increases in microbial numbers and changes in acid levels, and secondly whether organic acid resistant micro-organisms had to grow to cause the organic acid levels to decrease. Finally, the variation in carbon dioxide levels in the hay were examined as previously described.

The results obtained for this experiment are summarised in figs 35 - 40 and the temperature recordings in fig 41.

The temperature recordings show that the hay which had been stored for longer than twenty days had heated considerably to over 50°C, the heating having started almost immediately after the material had been packed into the dewar flasks. This rapid heating occurred even though the hay had been treated with 1.0% of the propionic acid: sorbic acid mixture, this probably being because very green hay at a high moisture level had been used. The hays which were examined after they had been in storage for 4, 8, 12, 16 and 20 days, were chosen because there had been some delay in the onset of their heating and it was hoped to correlate this delay with similar patterns in the other parameters of the hay being examined. Fig 41 also shows that none of the hays when examined had finished heating and therefore further changes could have taken place with longer storage periods.

Considering the actinomycete spore counts (fig 35), the numbers of thermophilic spores remained low until day 24 after which they increased to become a significant proportion of the total actinomycete spore count. The mesophilic spore numbers did not show such considerable changes although there was a four to five fold increase between hay stored for 8 and 12 days.

No cellulolytic thermophiles were detected, which agrees with the

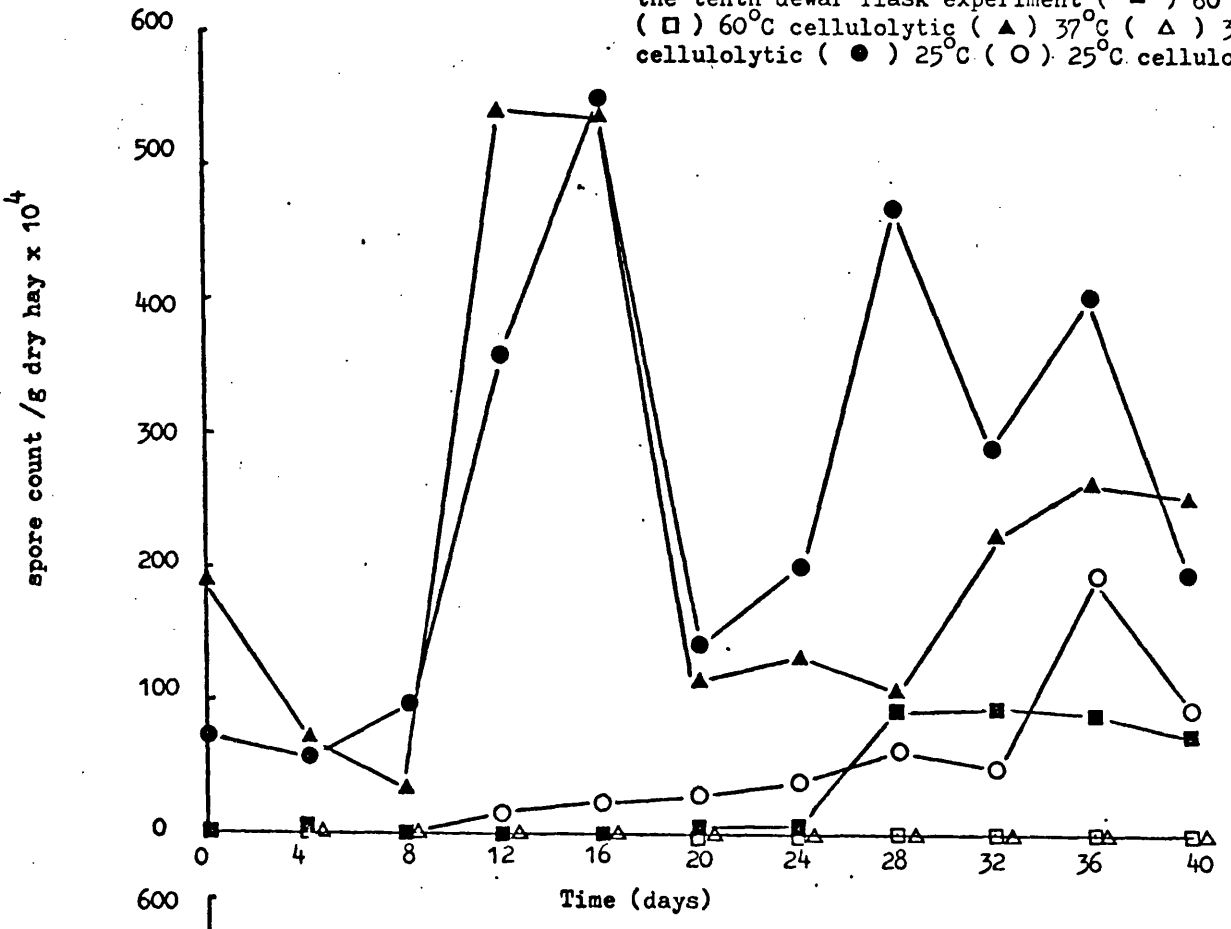
results of Fergus (1969), however cellulolytic mesophilic actinomycete spore numbers increased from less than 0.1 X 10^4/g dry hay to approximately 200 X 10^4/g dry hay after 36 days storage, suggesting that they became a more significant proportion of the total actinomycete spore numbers as the hay deteriorated in storage. The fungal spore counts (fig 36) and the bacterial counts (fig 37) followed a similar pattern to the actinomycete spore counts, with the thermophiles increasing after an initial storage period and the cellulolytic micro-organisms becoming more important after longer storage periods.

Fungal identification showed that the thermophiles were dominated by Mucor pusillus, Absidia ramosa and Aspergillus fumigatus for the first 16 days after which Thermomyces lanuginosa, Talaromyces dupontii and A. fumigatus were dominant along with some A. glaucus group spp. The most numerous mesophilic fungal species were Aspergillus spp, Penicillium spp and Mucor spp with occasional Fusarium spp being observed. The mesophilic, cellulolytic fungi consisted almost entirely of Scopulariopsis brevicaulis

Bacterial identification revealed the dominant mesophilic species were Bacillus spp, Pseudomonas spp and Micrococcus spp, whereas the thermophilic bacteria were dominated by Bacillus spp. The bacterial counts (fig 37), show how anaerobic bacteria remained a small proportion of the total bacterial flora.

In fig 38 alterations in the CO_2 and moisture levels of the hay during storage are shown. The CO_2 level in the hay initially increased rapidly to 2.2%, then decreased after which it fluctuated considerably. The moisture level of the hay remained constant for the first 20 days after which the hay dried slightly.

Fig 35 Actinomycete spore counts of hay from the tenth dewar flask experiment (■) 60°C (□) 60°C cellulolytic (▲) 37°C (△) 37°C cellulolytic (●) 25°C (○) 25°C cellulolytic.



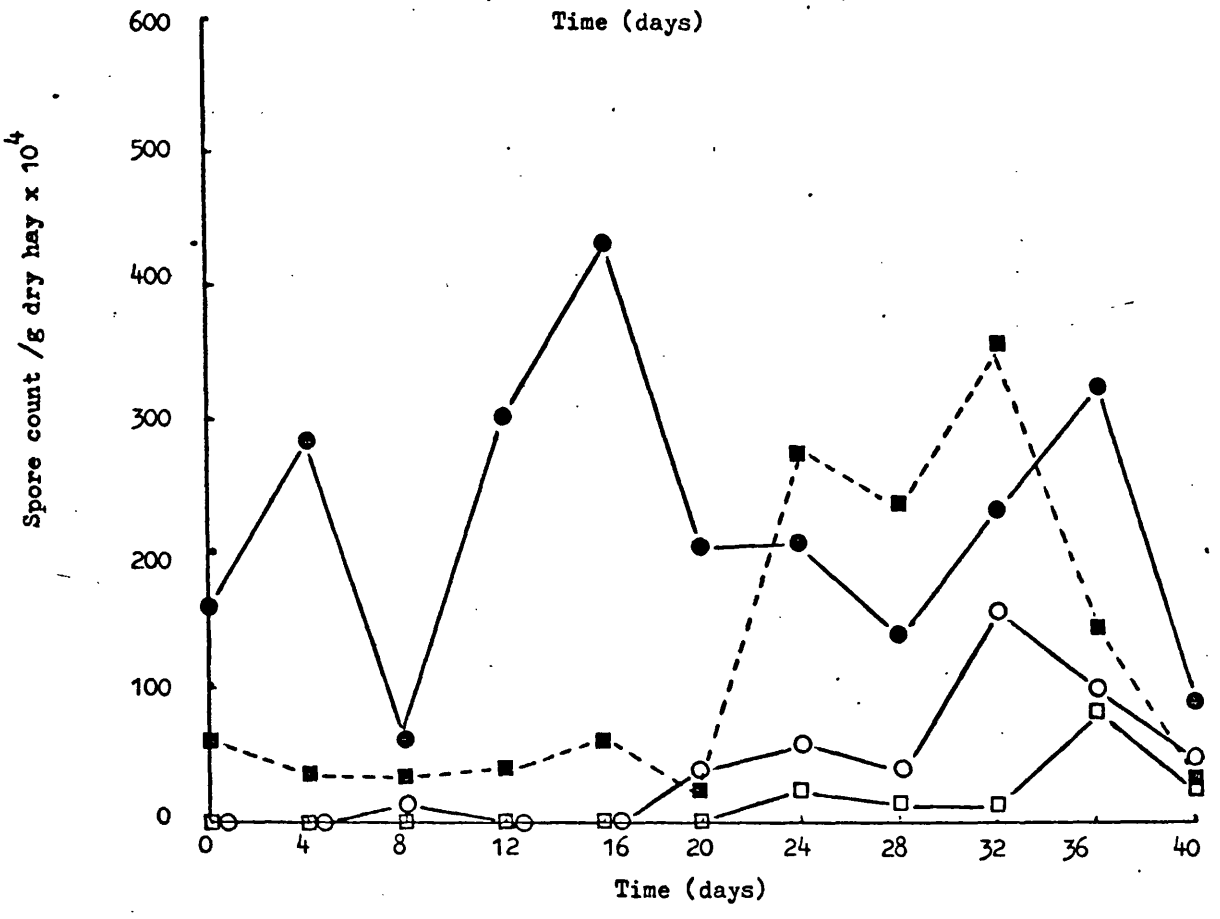


Fig 36 Fungal spore counts of hay from the tenth dewar flask experiment (■) 45°C (□) 45°C cellulolytic (●) 25°C (○) 25°C cellulolytic.

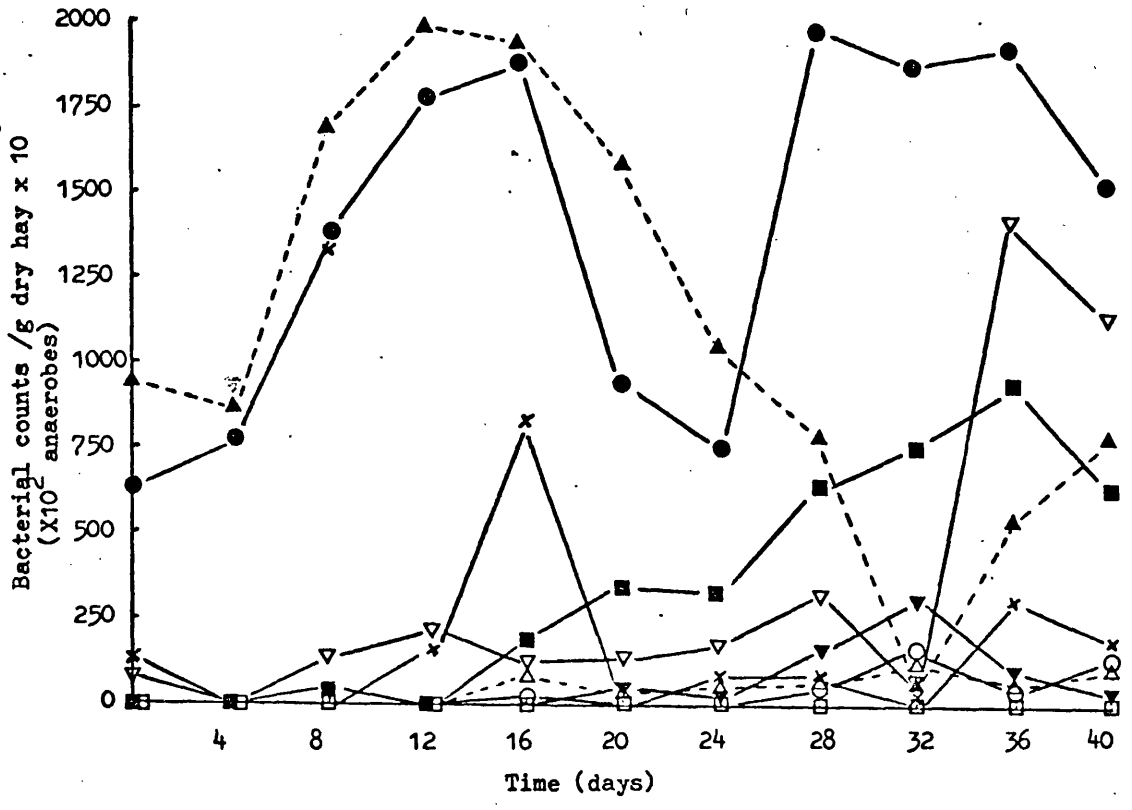


Fig 37 Bacterial counts of hay from the tenth dewar flask experiment (■) 60°C (□) 60°C cellulolytic (Δ) 60°C Anaerobic (●) 37°C (▼) 37°C cellulolytic (▽) 37°C Anaerobic (▲) 25°C (O) 25°C cellulolytic (×) 25°C anaerobic

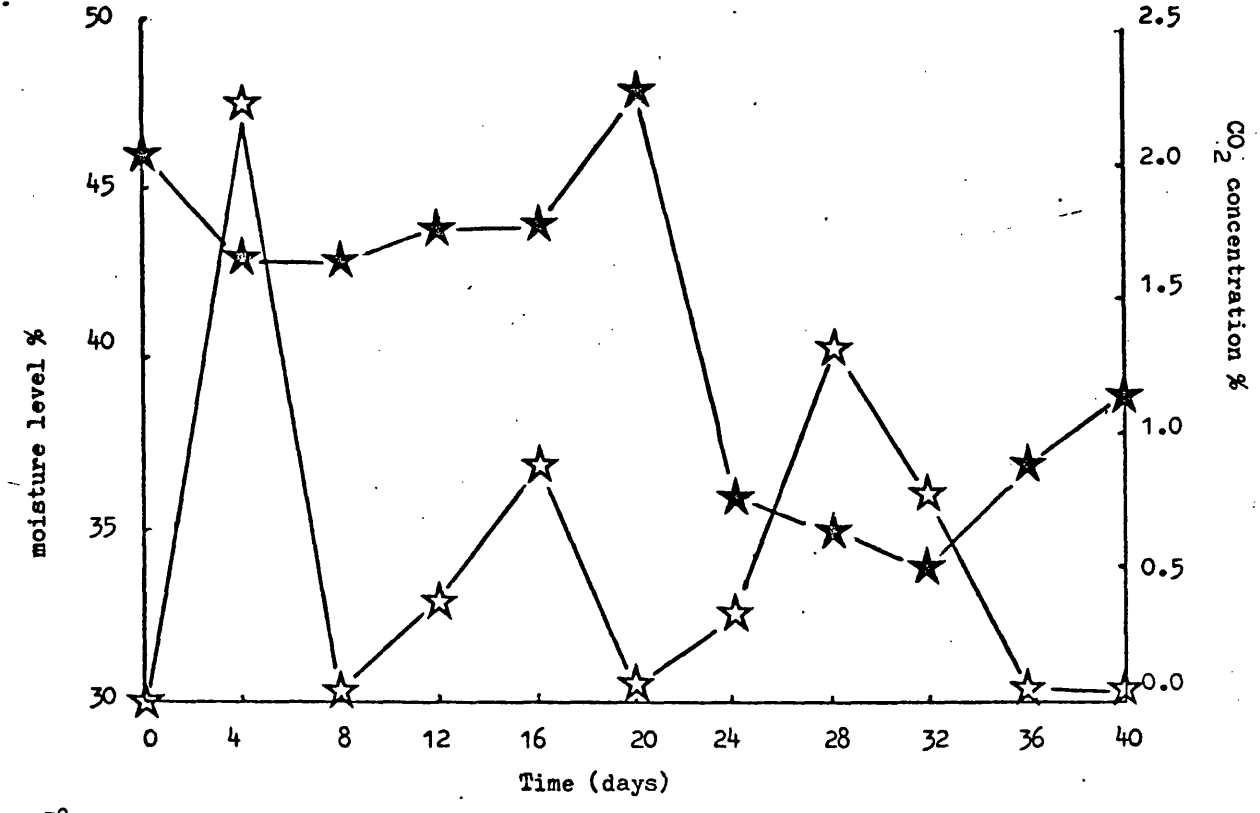


Fig 38 The moisture and CO_2 levels in the hay from the tenth dewar flask experiment. (★) moisture (☆) CO_2.

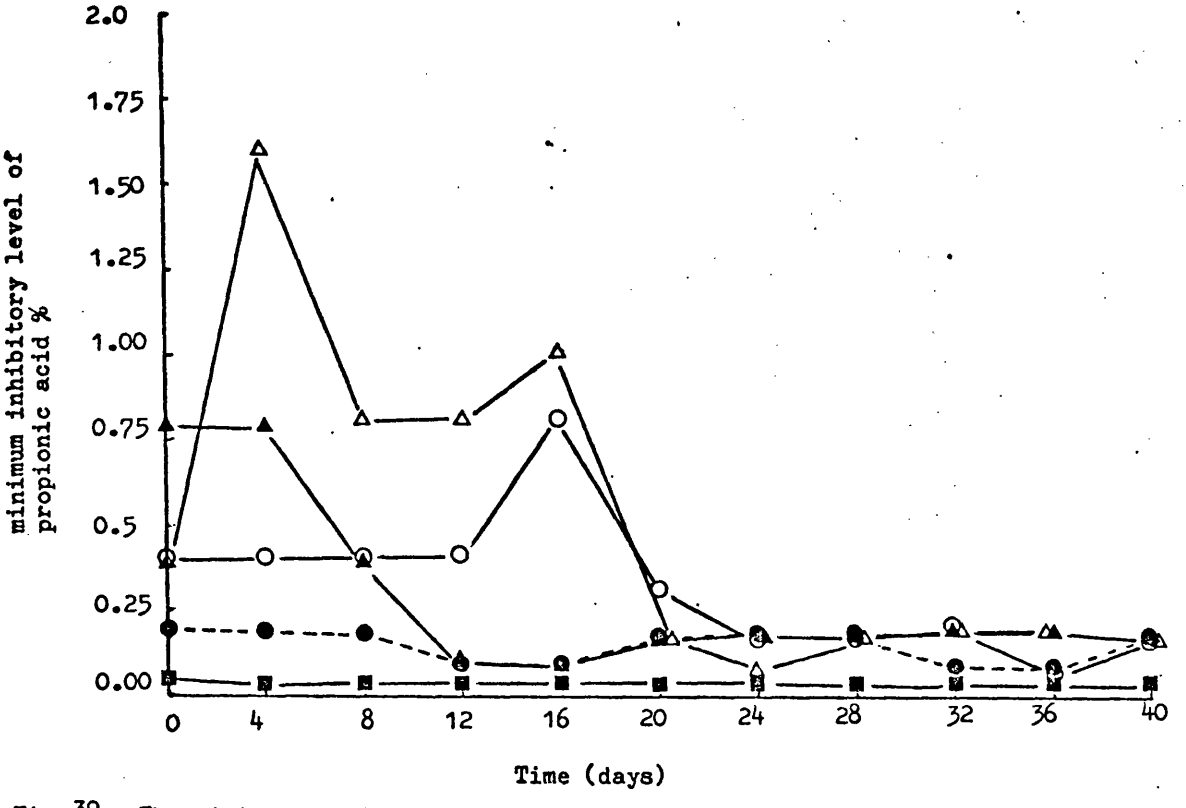


Fig 39 The minimum totally inhibitory level of propionic acid on the micro-organisms from hay in the tenth dewar flask experiment actinomycetes + bacteria. (■) at 60°C (○) at 37°C (△) at 25°C (●) Fungi at 45°C (◆) Fungi at 25°C.

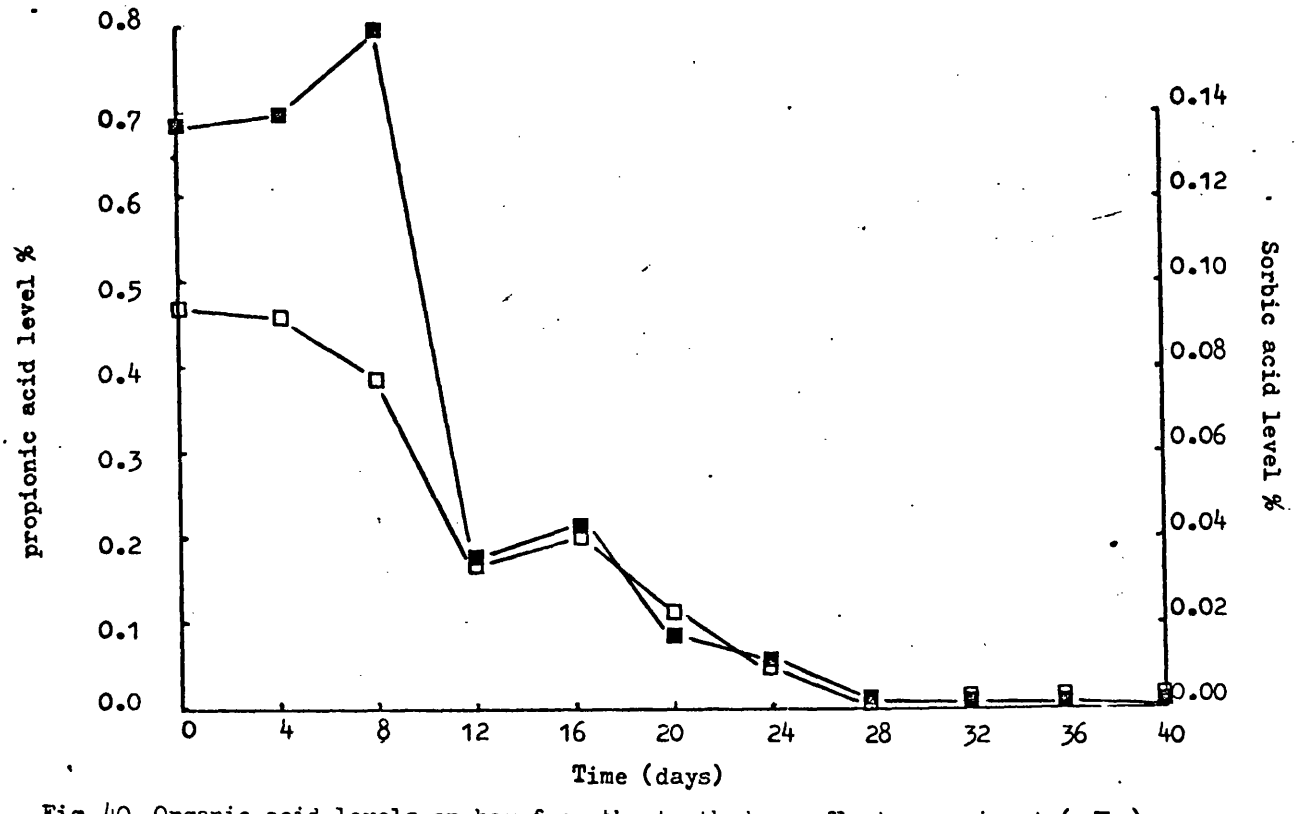


Fig 40 Organic acid levels on hay from the tenth dewar flask experiment (■) propionic acid (□) sorbic acid.

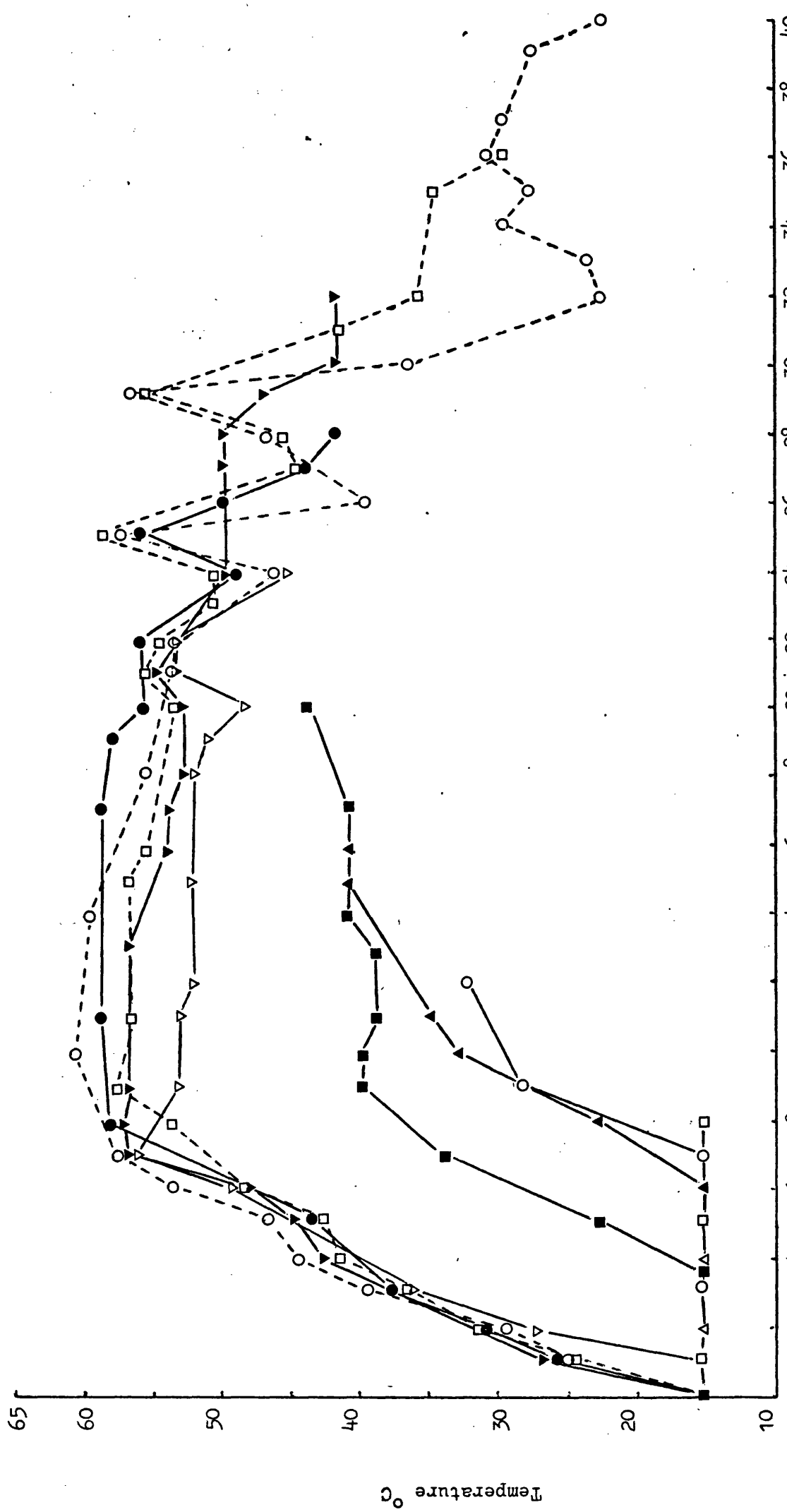


Fig 41 Temperature-time graph for the tenth dewar flask experiment (—△—) 4 days (—□—) 8 days (—○—) 12 days (—▲—) 16 days (—■—) 20 days (—▽—) 24 days (—●—) 28 days (—▼—) 32 days (-·□·-) 36 days (-·○·-) 40 days.

Fig (39) shows the minimum level of propionic acid in agar media which totally inhibited the growth of all the micro-organisms present in the hay. The resistance of the fungal species appears to have altered little during storage, however, some bacteria in the hay stored for less than 20 days appear to have been more resistant to propionic acid than any bacteria from hay stored for longer periods, suggesting that while high levels of propionic acid were present on the hay, the selective pressure created by this acid permitted the growth of propionic acid resistant micro-organisms and that when the acid levels decreased and this selective pressure was removed, non-resistant micro-organisms then outgrew the resistant micro-organisms.

The organic acid levels (fig 40) decreased on the hay which had been stored for longer than eight days, suggesting that while the hay did not heat, the acid levels remained constant, whereas once the hay commenced heating the organic acid levels rapidly decreased. Finally there appeared to be no difference between the persistance of the propionic acid and that of the sorbic acid on the hay.

d) The use of field trials

i) The first field trial

The objective of this field trial was to study the changes in hay baled containing 42%, 19% and 15% moisture, which had been treated with 0, 0.1%, 0.2%, 0.4% and 0.8% of the commercial hay preservative, marketed by Feed Services Ltd., and known as Hay Shield, which contained approximately 30% propionic acid.

The results obtained from this field trial are summarised in table 31 and the temperature recordings are shown in figs 42, 43 and 44.

Considering the 42% moisture hay, the untreated and all the treated

hays heated (fig 42), and their microbial spore counts increased considerably. All the treatments significantly ($P < .05$) reduced the increase in thermophilic actinomycete spore counts but the 0.1% and 0.2% Hay Shield treatments reduced the increase in fungal spore numbers more than the 0.4% and 0.8% treatments.

The 15% moisture hay heated little (fig 43) although all the treatments caused a small but significant ($P < .05$) decrease in the mesophilic actinomycete and thermophilic fungal spore numbers.

All the treatments prevented heating of the 19% moisture hay, the untreated hay heating to 35°C (fig 44). Microbial spore count changes were too limited to allow the treatments to show any significant ($P < .05$) effects.

ii) The second field trial

From the results of the first field trial it appears that Hay Shield was not an effective hay preservative, and therefore in further experiments, mixtures with improved formulations were used.

In the second dewar flask experiment (Table 23 fig 26), the addition of sorbic acid had improved the activity of Hay Shield and therefore in the second field trial this mixture was compared with the most effective organic acid mixture in agar media (tables 15 -18), this being the propionic acid: n-butyric acid: sorbic acid (45:45:10 w/w/w) formulation. Formaldehyde was used with this mixture, this compound being added in order to determine whether it would act synergistically with the organic acids.

These treatments were applied at rates of 0.25% 0.5% and 0.75%,

Treatment	Application Rate %	Moisture content at boiling %	Heating max temp °C	Heating degree days above 25°C	Microbial spore counts X 10^4/g of hay: Actinomycetes incubated at 60°C	Actinomycetes incubated at 37°C	Actinomycetes incubated at 25°C	Fungi incubated at 45°C	Fungi incubated at 25°C	Visual assessment of hay after storage	reference for statistical analysis
Initial hay at cutting	-	-	-	-	10	10	81	10	2.8	-	
Initial hay at baling for 42% moisture hay	-	-	-	-	0.6	6.1	22	3.3	1.1	-	
Initial hay at baling for 15% and 19% moisture hay	-	-	-	-	2.4	2.3	67	2.0	0.8	-	
Untreated	-	42	65	440	50	10		32	36	mouldy	1A
Hay Shield+	0.1	42	65	468	30	25		14	16	mouldy	1B
Hay Shield	0.2	42	65	350	14	8		4	14	mouldy	1C
Hay Shield	0.4	42	59	339	28	44		55	42	mouldy	1D
Hay Shield	0.8	42	54	267	24	38		67	98	mouldy	1E
Untreated	-	15	23	0	4.2	11.4		4.2	7.8	Good	2A
Hay Shield	0.1	15	23	0	2.1	3.4		1.5	6.6	Good	2B
Hay Shield	0.2	15	23	0	2.7	2.0		1.0	3.6	Good	2C
Hay Shield	0.4	15	22	0	1.8	0.8		0.6	2.1	Good	2D
Hay Shield	0.8	15	21	0	2.4	1.6		0.9	4.4	Good	2E
Untreated	-	19	35	155	2.6	0.8		0.8	5.1	Good	3A
Hay Shield	0.1	19	24	0	2.0	0.6		0.6	2.6	Good	3B
Hay Shield	0.2	19	23	0	2.7	0.7		0.5	2.4	Good	3C
Hay Shield	0.4	19	23	0	2.6	0.5		0.4	2.3	Good	3D
Hay Shield	0.8	19	23	0	1.9	0.4		0.4	5.0	Good	3E

Statistical analysis (P< .05)

Moisture level one

actinomycetes incubated at 60°C - 1A > 1B, 1C, 1D and 1E.

actinomycetes incubated at 37°C - No differences.

Fungi incubated at 45°C - 1A 1D, 1E > 1B 1C.

Fungi incubated at 25°C - 1A, 1E > 1B 1C, 1D.
1D > 1B, 1C.

Moisture level two

Actinomycetes incubated at 60°C - No differences.

Actinomycetes incubated at 37°C - 2A > 2B, 2C, 2D, 2E.

Fungi incubated at 45°C - 2A > 2B, 2C, 2D 2E.

Fungi incubated at 25°C - No differences.

Moisture level three

No significant changes

Table 31 - Results for the first field trial before and after 32 days storage.

* Moisture level one = hay baled containing 42% moisture
Moisture level two = " " " 15% "
Moisture level three = " " " 19% "

< = Less than

+ Hay Shield = a commercial hay preservative marketed by Feed Services Ltd, and containing approximately 30% propionic acid.

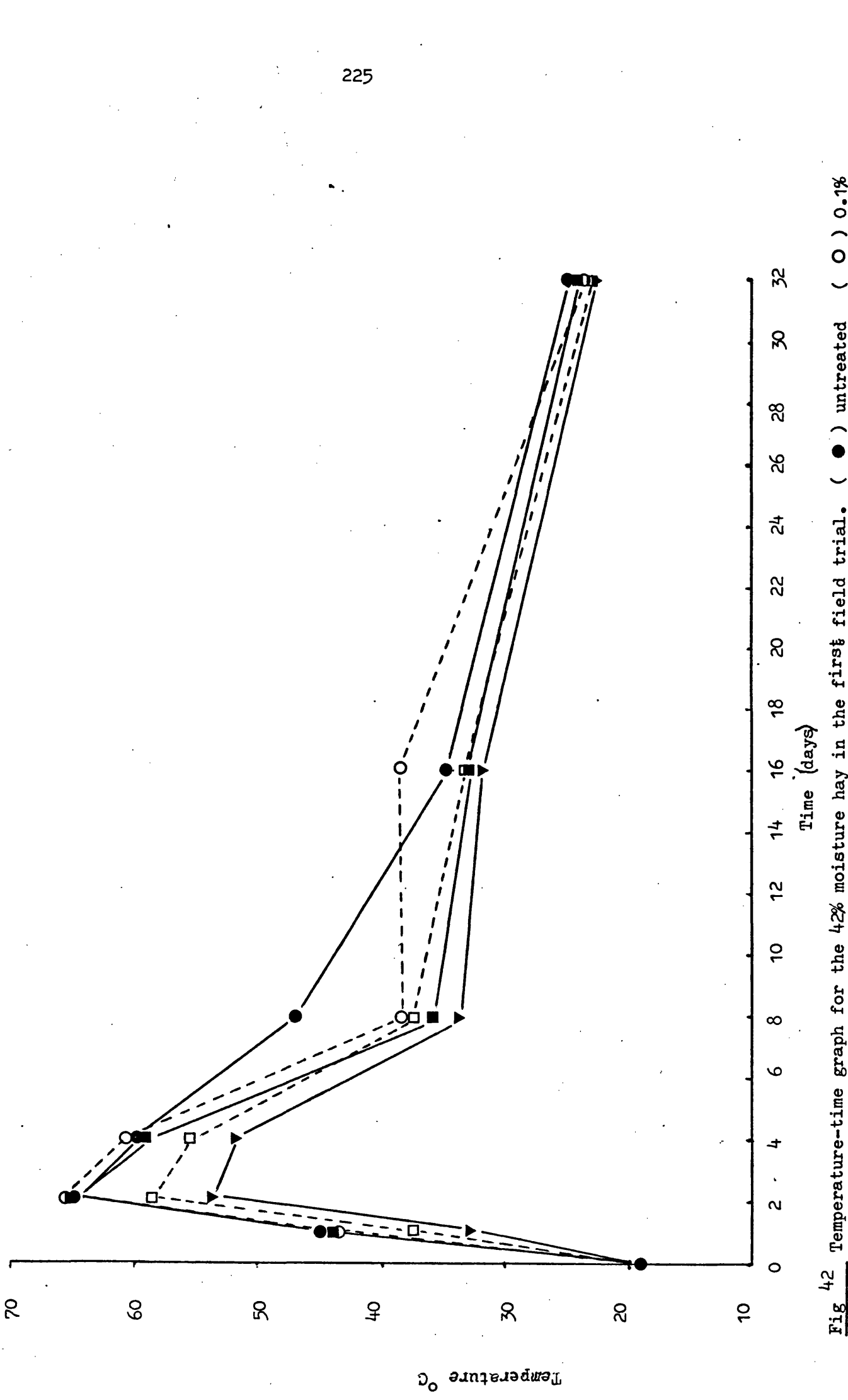


Fig 42 Temperature-time graph for the 42% moisture hay in the first field trial. (●) untreated (O) 0.1% Hay shield (H.S.) (■) 0.2% H.S. (□) 0.4% H.S. (▼) 0.8% H.S.

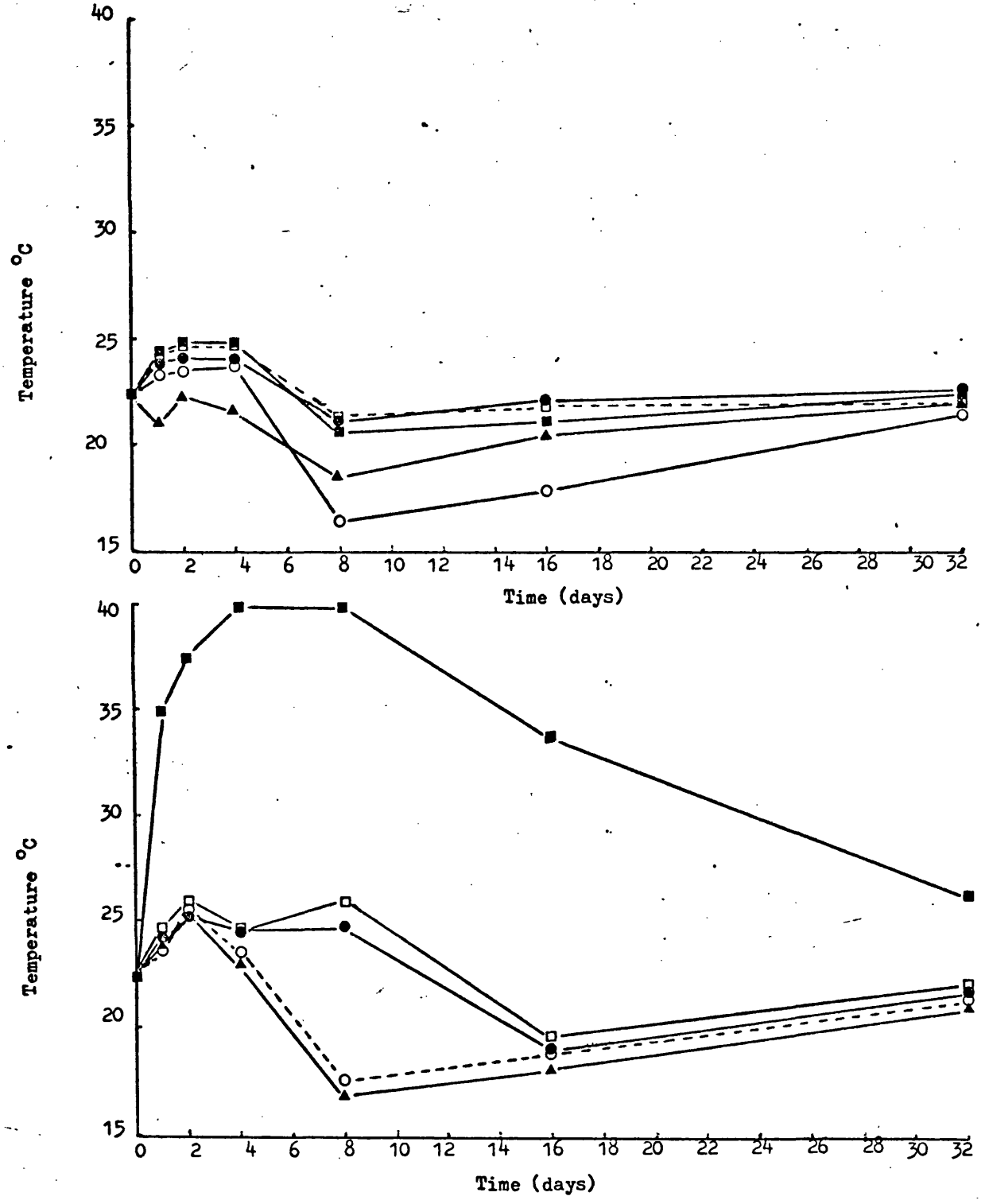


Fig 43 Temperature time graph for the 15% moisture hay in the first field trial.

Fig 44 Temperature time graph for the 19% moisture hay in the first field trial.

(■) Untreated (□) 0.1% Hay Shield (H.S.) (●) 0.2% H.S. (○) 0.4% H.S. (▲) 0.8% H.S.

to hay with an initial moisture content of 19%.

The results for this field trial are summarised in table 32 and the temperature recordings are shown in figs 45 and 46.

In this experiment the hay was dry and therefore both heating and microbial spore increases were very limited, although the thermophilic actinomycete spore count increased considerably during storage. These increases, however, were unaffected by any of the treatments. The untreated and treated hay were considered, by visual examination, to be in good condition after storage and therefore the treatments did not have the opportunity to show any significant effects. Despite the fact that the hay did not deteriorate, the applied organic acid levels fell during the 64 days storage to below 0.01% on all the treated hays.

iii) The third field trial

Because the hay in the second field trial was too dry to deteriorate, in the third field trial the same chemical treatments were used, at application rates of 0.25% and 0.5%, however, damper hay (44% moisture) was treated.

The results are summarised in table 33, the organic acid levels on twelve samples from one treated bale are shown in table 34 and temperature recordings are shown in fig 47.

Because the hay was baled with a high moisture level the untreated material heated rapidly to 60°C and the treated hays heated to between

Treatment	Application Rate %	Moisture content at baling %	organic acid level % At baling propionic	At baling n-butyric	After storage propionic	After storage n-butyric	Heating max degree temp °C	Heating days above 20°C	Microbial spore counts X 10^4/g hay Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	Reference for statistical analysis	Visual assessment of hay after storage
Initial Hay	-	-	-	-	-	-	-	-	<0.1	40	20	30	300	-	-
Untreated	-	19	<0.01	<0.01	<0.01	<0.01	38	207	11	124	71	70	130	A	Good
Hay Shield:sorbic acid 96:4 (w/w)	0.25	19	0.017	<0.01	<0.01	<0.01	35	245	14	54	22	82	120	B	Good
Hay Shield:sorbic acid 96:4 (w/w)	0.50	19	0.018	<0.01	<0.01	<0.01	31	85	13	64	26	76	180	C	Good
Hay Shield: sorbic acid 96:4 (w/w)	0.75	19	0.023	<0.01	<0.01	<0.01	34	119	4	36	18	60	180	D	Good
propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w)(Mixture A)	0.25	19	0.043	0.052	<0.01	<0.01	29	74	11	130	61	36	180	E	Good
mixture A	0.50	19	0.052	0.061	<0.01	<0.01	28	86	7	90	67	45	220	F	Good
mixture A	0.75	19	0.074	0.083	<0.01	<0.01	26	44	11	94	56	45	150	G	Good
mixture A: 40% formaldehyde in water 7:3 (v/v) mixture B	0.25	19	0.037	0.043	<0.01	<0.01	30	85	79	80	11	52	100	H	Good
mixture B	0.50	19	0.048	0.054	<0.01	<0.01	32	124	9	50	17	51	110	I	Good
mixture B	0.75	19	0.058	0.067	<0.01	<0.01	33	172	7	53	20	44	90	J	Good

Table 32 - Results for the second field trial - before and after 64 days storage.

* The organic acids were extracted by soaking the hay in 0.6N H_2SO_4 for seven days

Statistical analysis ($P < .05$) - No significant differences.

< = Less than

(■) 0.25% O.A. (□) 0.75% O.A. (●) 0.25% O.A. with formaldehyde (O.A.F.)
(○) 0.5% O.A.F. (▼) 0.75% O.A.F. (19% moisture hay).

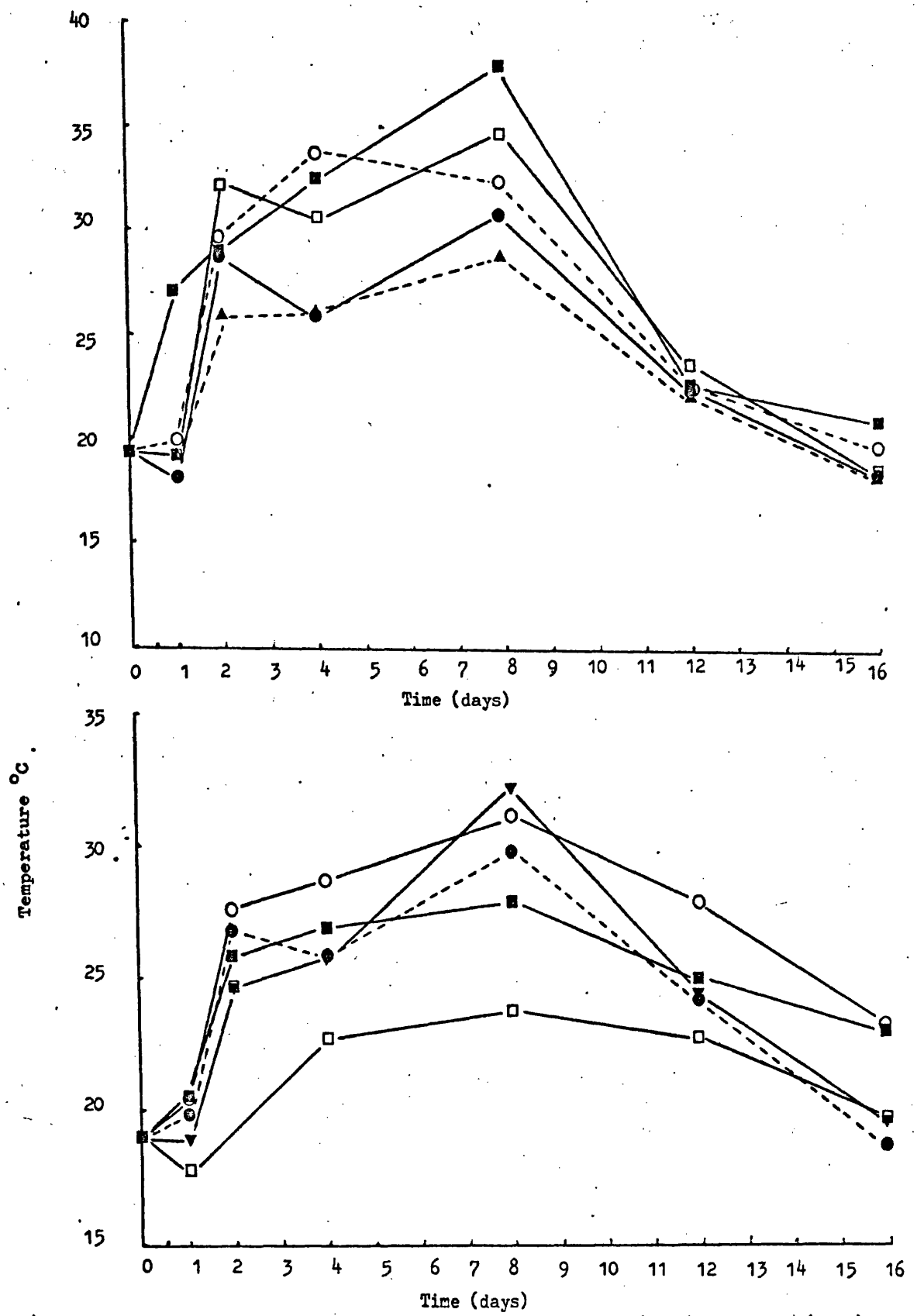


Fig 45 Temperature time graphs for the second field trial (■) untreated (□) 0.25% Hay shield + sorbic acid (H.S.) (●) 0.5% H.S. (O) 0.75% H.S. (▲) 0.25% organic acid mixture (O.A.)

Fig 46 (■) 0.5% O.A. (□) 0.75% O.A. (●) 0.25% O.A. with formaldehyde (O.A.F.) (O) 0.5% O.A.F. (▼) 0.75% O.A.F. (19% moisture hay).

50°C and 60°C although the propionic acid; n-butyric acid; sorbic acid mixture both with and without formaldehyde, did delay the temperature maxima by 2 - 3 days.

The thermophilic actinomycete spore numbers only increased significantly during storage in the untreated and 0.25% of Hay Shield containing 4% sorbic acid treatments, whereas the actinomycete spores incubated at 37°C and the thermophilic fungal spore numbers increased considerably in all the hays, although by more in the untreated and 0.25% of Hay Shield containing 4% sorbic acid treated hays. The spores, incubated at 25°C, numbers tended to decrease in the hay during storage except in the untreated hay, and this was possibly because the combination of heat and organic acids rendered some of these spores non-viable.

Although none of the treatments reduced heating, all of them, except the 0.25% Hay Shield containing 4% sorbic acid, reduced the final thermophilic spore counts which suggested that these hays could have been less hazardous to handle and feed. The spore counts were in agreement with visual estimates of moulding of the hay made after three months storage.

The organic acid levels of the twelve samples taken immediately after baling, from a bale of hay treated with 0.5% of the propionic acid; n-butyric acid: sorbic acid mixture, are shown in table 34. They indicate the poor preservative distribution being obtained within the bales which is substantiated by similar results from the fourth and fifth field trials.

Treatment	Application rate %	moisture content at baling	Heating max temp °C	Heating degree days above 20°C	Microbial spore counts x 10^4/g hay after 40 days storage					Microbial spore counts x 10^4/g hay after three months storage					Reference for statistical analysis	Visual assessment of hay after storage
					Actinomycetes incubated at			Fungi incubated at		Actinomycetes incubated at			Fungi incubated at			
					60°C	37°C	25°C	45°C	25°C	60°C	37°C	25°C	45°C	25°C		
Initial hay	-	-	-	-	2	3.5	7.0	8.1	9.2	2	3.5	7.0	8.1	9.2	-	-
Untreated	-	44	59	298	250	220	250	210	270	131	880	920	690	940	A	Very mouldy
Hay shield:sorbic acid 96:4 (w/w)	0.25	44	64	355	390	370	260	190	250	139	380	450	440	730	B	Very mouldy
Hay shield:sorbic acid 96:4 (w/w)	0.50	44	60	313	240	270	350	430	290	4	140	160	280	490	C	Very mouldy in patches
propionic acid: n-butyric acid: sorbic acid 45:45:10 (w/w/w) mixture A	0.25	44	54	351	180	180	110	190	210	24	190	120	230	380	D	Mouldy
mixture A	0.50	44	54	302	210	220	60	80	1100	3	120	70	200	380	E	little moulding
mixture A: 40% formaldehyde in water 7:3 (v/v) mixture B	0.25	44	49	278	120	380	280	750	850	2	90	70	260	320	F	Bad patches of moulding
mixture B	0.50	44	58	329	140	460	350	380	610	10	80	70	130	220	G	slight moulding

Table 33 - Results for the third field trial before storage and after 40 days and three months storage.

Statistical analysis ($P < .05$)

After 40 days storage - No significant differences

After 3 months storage - Actinomycetes incubated at 60°C A,B > C,D,E,F,G.
" " " 37°C A > B,C,D,E,F,G.
" " " 25°C A > B,C,D,E,F,G.
B > C,D,E,F,G.
C > E,F,G.

Fungi incubated at 45°C A,B > C,D,E,F,G.
" " " 25°C A > C,D,E,F,G.
B > D,E,F,G.
C > F,G.
A,B,C,D,E,F > G.

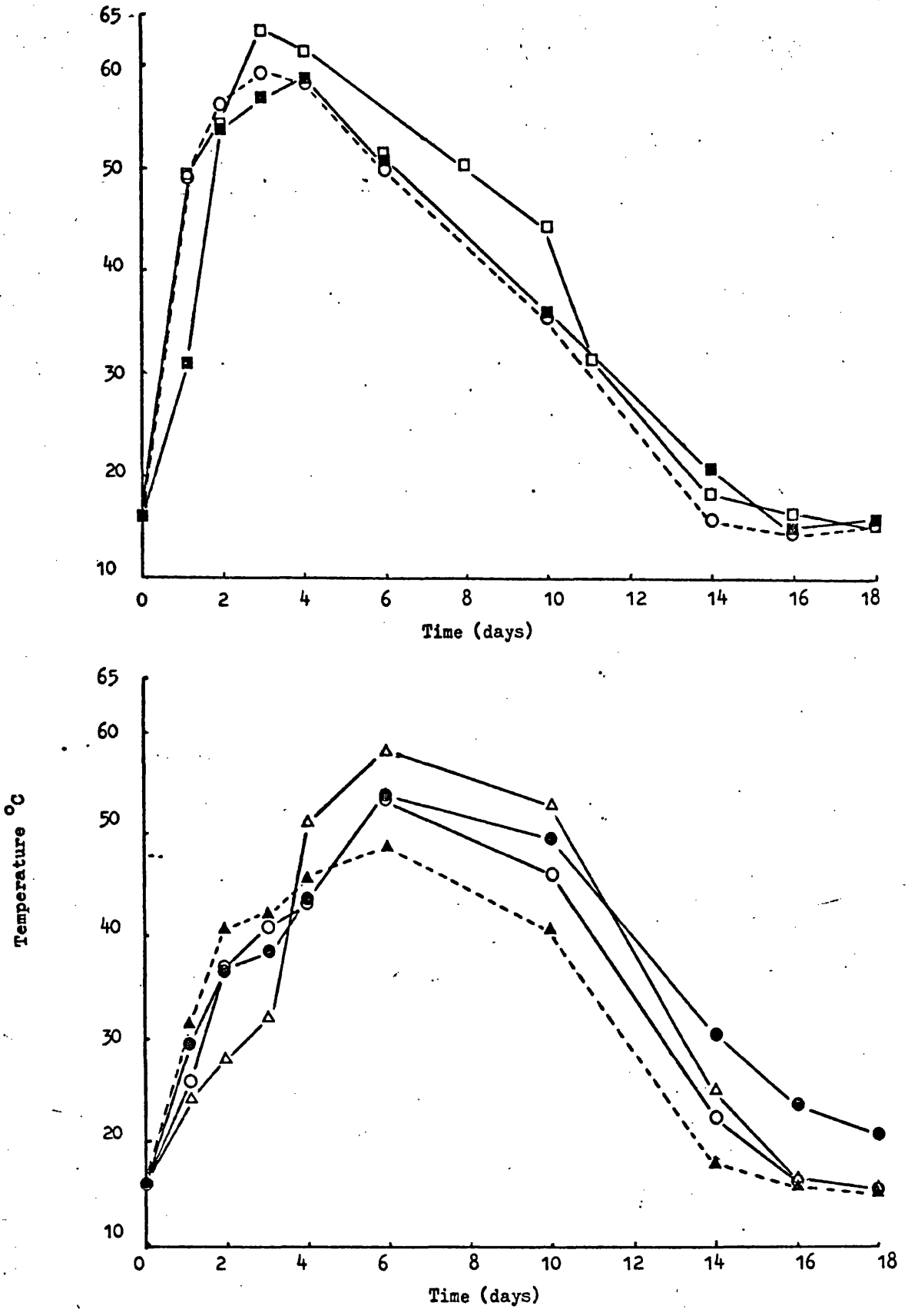


Fig 47 Temperature-time graphs for the third field trial (—■—) untreated (—□—) 0.25% Hay Shield + sorbic acid (H.S.) (--O--) 0.5% H.S. (—●—) 0.25% Organic acid mixture (O.A) (—O—) 0.5% O.A. (--▲--) 0.25% O.A. & formaldehyde (—△—) 0.5% O.A. + formaldehyde. (44% moisture hay)

Sample	Propionic acid level %	n-butyric acid level %	Sample	propionic acid level %	n-butyric acid level %
1	0.120	0.107	7	0.023	0.024
2	0.082	0.093	8	0.066	0.068
3	0.027	0.041	9	0.040	0.038
4	0.026	0.032	10	0.036	0.044
5	0.019	0.021	11	0.044	0.052
6	0.066	0.057	12	0.057	0.066

Table 34 - Organic acid levels on twelve random samples taken from a bale of hay, immediately after baling, which had been treated with 0.5% of a propionic acid: n-butyric acid: sorbic acid mixture 45:45:10 (w/w/w).
The organic acids were extracted by soaking the hay in 0.6N H_2SO_4 for seven days.

iv) The fourth field trial

The results in the third field trial suggested that application rates greater than 0.5% were necessary for control, and that the Hay Shield: sorbic acid mixture was not as effective as the organic acids. N-butyric acid was considered impractical as a hay preservative due to its high price and unpleasant smell and formaldehyde was rejected due to its apparent inactivation when applied to hay.

Therefore, in the fourth field trial, propionic acid and a propionic acid: sorbic acid mixture (9:1 w/w) were applied at rates of 0.5% 1.0% and 2.0% to hay with an initial moisture content of 32%.

The changes in the hay were followed to determine whether sorbic acid had improved the effectiveness of propionic acid as a hay preservative and the results are summarised in table 35. Tables 36, 37 and 38 show the determined organic acid levels on hay samples taken from treated bales immediately after baling and after six weeks and three months storage, in order to demonstrate the preservative distribution within the bales and their persistence on the hay.

Temperature recordings are shown in fig 48.

In this experiment the untreated hay heated to 50°C after eight days storage. The treated hays heated to at least 40°C and upon visual examination, after three months storage, appeared dusty, except for the hay treated with 2.0% of the propionic acid; sorbic acid mixture which had not heated above 30°C and had the visual appearance of good hay after three months storage.

Ten bales for each treatment, were prepared and stacked together and two of the bales stored in the central part of each pile were used for temperature recordings. One of these bales was carefully broken open after six weeks and five samples were taken for microbial spore counts, whereas a third central bale was used for the three month spore estimations. When all the bales from any one of the treatments were examined after three months storage they all had a similar appearance except for the bales which had been treated with 1.0% of the propionic acid; sorbic acid mixture, where the two bales which had been used for temperature measurements appeared very mouldy, but the remaining eight contained only a little patchy moulding. A possible explanation for this is that, of the ten bales treated with 1.0% of the propionic acid: sorbic acid mixture, two came from a damp patch in the field and these two bales were used for the temperature recordings and the six week microbial counts shown in table 35. This explanation would account for both the apparent increase in heating associated with the 1.0% propionic acid: sorbic acid mixture treatment and the large difference in microbial spore counts for this hay after six weeks storage and after three months storage (table 35), the latter counts being made from a third drier hay bale.

The thermophilic actinomycete spore numbers increased considerably in the untreated and the 0.5% propionic acid treated hay and significantly ($P < .05$) smaller increases occurred in the remaining treated hays during three months storage. However, both the mesophilic actinomycete and the thermophilic fungal spore counts changed little during the three months storage, whereas the mesophilic fungal spore numbers increased in all the treated and untreated hays but only small increases

occurred in the hay treated with 2.0% of the propionic acid, sorbic acid mixture.

These results suggest that for hay baled containing 32% moisture, a treatment of 2.0% of the propionic acid; sorbic acid mixture would be sufficient to prevent it from deteriorating.

The high coefficients of variation for the results shown in table 36, demonstrate the uneven preservative distribution that was obtained in each treated hay bale examined immediately after application and there was no evidence to support the hypothesis that increasing the application rates would give a more even distribution of preservative.

A comparison of the results in tables 37 and 38 show that the organic acid levels on the hay were apparently no lower and occasionally even higher after three months storage than after six weeks storage. The fact that higher levels were sometimes recorded after three months storage, for example with the 0.5% and 1.0% propionic acid treated hays, was possibly, because either the bales examined after three months were drier at the time of baling and therefore had deteriorated less than the hay examined after six weeks or they had received higher levels of propionic acid at the time of baling.

These results also suggest that the lowering of the organic acids levels on the hay during storage occurred when the hay heated, and that when the heating phase was completed the acid levels remained relatively constant. The 2.0% propionic acid; sorbic acid mixture

treated hay bales had a strong smell of propionic acid when they were broken open after three months storage and the results in table 38 suggest that approximately 50% of the applied propionic acid and sorbic acid were still present on this hay after this storage period.

v) The fifth field trial

This experiment was designed to determine whether propionic acid applied at a rate of 2.0% was a more effective hay preservative than a propionic acid: water mixture 1:1 (v/v) which was also applied at a rate of 2.0%. It was considered possible that higher application rates would have given a more even distribution of the preservative and therefore by diluting the propionic acid with water and applying it at a higher rate, the preservative properties of the acid may have been improved.

The temperature recordings for this field trial (fig 49) show some initial heating occurred although this was thought to be due to plant respiration rather than microbial respiration, because firstly, the hay was considered too dry (22% moisture) for any significant microbial growth to have taken place and secondly, heating due to microbial activity does not decrease after the first day as can be seen if the temperature recordings for the first four field trials are examined. Due to this considered lack of microbial activity, microbial counts were not made for this hay.

Table 39 shows estimated propionic acid levels on samples of hay taken from treated bales immediately after baling and after five weeks storage. The coefficients of variation are higher than for the fourth

Treatment	Application rate %	Moisture content at baling % Initial	Moisture content at baling % Final*	Heating max temp	Heating degree days above 25°C	Microbial spore counts x 10^4/g hay after six weeks storage: Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	Microbial spore counts X 10^4/g hay After three months storage: Actinomycetes incubated at 60°C	37°C	25°C	Fungi incubated at 45°C	25°C	Reference for statistical analysis	Visual assessment of hay after 3 months storage
Initial Hay	-	-	-	-	-	0.1	12	3.6	5.2	5.8	0.1	12	3.6	5.2	5.8	-	-
Untreated	-	32	14.5	51	271	73	19	1.1	22	620	68	9.8	12	14	600	A	Dusty
propionic acid	0.5	32	15.7	50	271	21	0.7	0.4	24	577	48	32	37	12	620	B	Very mouldy in patches
propionic acid	1.0	32	18.1	41	89	11	0.1	0.16	92	605	6.2	8.8	6	3	480	C	Mouldy in patches
propionic acid	2.0	32	19.2	43	339	12	0.2	0.1	31	590	0.2	22	6	62	534	D	little moulding
propionic acid: sorbic acid 9:1 (w/w)	0.5	32	16.7	42	78	1.7	1.1	0.6	19	610	0.2	6	3.6	12	571	E	Slight moulding
propionic acid: sorbic acid 9:1 (w/w)	1.0	32	17.0	60	467	82	178	341	105	587	0.7	7.2	2.4	11	592	F	Slight moulding
propionic acid: sorbic acid 9:1 (w/w)	2.0	32	22.0	30	30	0.1	24	15	11	469	1.2	4	1.1	3.8	373	G	Good hay strong smell of propionic acid

Table 35 - Results for the fourth field trial before storage and after six weeks and three months storage.

* After three months storage.

Statistical analysis after six weeks storage (P< .05)

Actinomycetes incubated at	60°C	A,F >B,C,D,E,G. A,B,C,D,E,F >G.
"	37°C	F > A,B,C,D,E,G.
"	25°C	F > A,B,C,D,F,G.
Fungi	45°C	No differences
"	25°C	A,B,C,D,E,F, >G.

Statistical analysis after three months storage (P< .05)

Actinomycetes incubated at	60°C	A> B,C,D,E,F,G. B> C,D,E,F,G.
"	37°C	No differences
"	25°C	No differences
Fungi	45°C	No differences
"	25°C	AB,D,E,F >G.

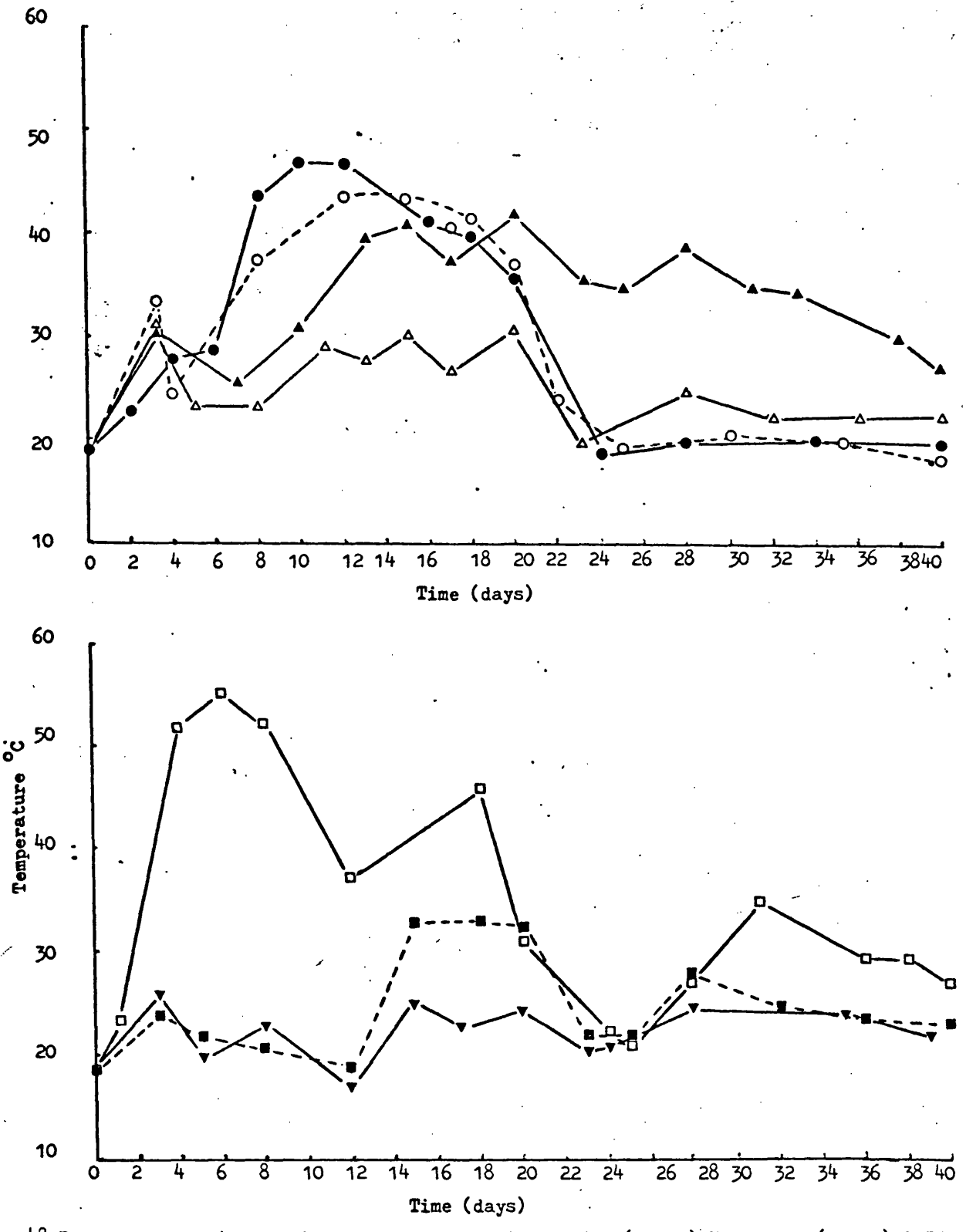


Fig 48 Temperature - time graphs for the fourth field trial (●) Untreated (o) 0.5% propionic acid (P.A.) (△) 1.0% P.A. (▲) 2.0% P.A. (■) 0.5% P.A. with sorbic acid (P.S.) (□) 1.0% P.S. (▼) 2.0% P.S. (32% moisture hay)

Determined organic acid levels * % on wet hay									
0.5% propionic acid	1.0% propionic acid	2.0% propionic acid	0.5% propionic acid : sorbic acid 9:1 (w/w)		1.0% propionic acid :sorbic acid 9:1 (w/w)		2.0% propionic acid :sorbic acid 9:1 (w/w)		Treatment
propionic acid	propionic acid	propionic acid	propionic acid	sorbic acid	propionic acid	sorbic acid	propionic acid	sorbic acid	Acid determined
0.29	0.06	0.40	0.05	0.004	0.50	0.060	1.59	0.190	
0.14	0.21	0.21	0.21	0.025	0.72	0.066	0.54	0.041	Acid
0.19	0.64	0.64	0.16	0.005	0.75	0.046	0.15	0.008	levels
0.14	0.26	0.26	0.02	0.004	0.27	0.014	0.34	0.021	% by weight
0.08	0.32	0.32	0.02	0.004	0.81	0.049	0.38	0.008	on the
0.05	0.22	0.22	0.03	0.005	0.27	0.015	0.87	0.252	hay
0.14	0.06	0.06	0.59	0.057	0.56	0.024	0.11	0.020	samples
0.50	0.05	0.05	0.77	0.035	0.14	0.004	0.31	0.181	
0.11	0.13	0.13	0.74	0.017	0.67	0.048	0.64	0.303	
0.26	0.14	0.14	0.74	0.018	0.16	0.004	0.34	0.050	
0.02	0.28	0.28	0.03	0.006	0.58	0.030	0.22	0.030	
0.02	0.16	0.16	0.34	0.020	0.59	0.017	0.52	0.271	
0.16	0.21	0.24	0.31	0.017	0.50	0.031	0.50	0.110	Mean
32%	21%	12%	62%	34%	50%	31%	25%	55%	% recovery
82	74	65	97	92	45	67	77	99	Coefficient of Variation

Table 36 - Organic acid level determinations on samples from bales of hay treated in the fourth field trial - Immediately after baling.

* The organic acids were extracted by soaking the hay in 0.6N H_2SO_4 for seven days.

Determined organic acid levels * % on wet hay									
0.5% propionic acid	1.0% propionic acid	2.0% propionic acid	0.5% propionic acid: sorbic acid 9:1 (w/w)		1.0% propionic acid: sorbic acid 9:1 (w/w)		2.0% propionic acid: sorbic acid 9:1 (w/w)		Treatment
P.A.	P.A.	P.A.	P.A.	S.A.	P.A.	S.A.	P.A.	S.A.	Acid Determined
0.021	0.104	0.83	0.300	0.005	0.004	0.008	0.85	0.075	Acid levels % by weight on the hay samples
0.014	0.024	0.75	0.225	0.005	0.008	0.012	0.85	0.108	
0.013	0.008	0.83	0.060	0.006	0.033	0.019	0.84	0.204	
0.007	0.038	0.88	0.020	0.008	0.092	0.016	1.10	0.208	
0.007	0.023	0.69	0.007	0.012	0.088	0.016	0.60	0.067	
0.016	0.039	0.73	0.012	0.008	0.100	0.005	0.75	0.138	
0.004	0.019	0.055	0.160	0.006	0.016	0.005	1.28	0.128	
0.008	0.010	≤0.001	0.095	0.010	0.233	0.005	2.25	0.833	
0.003	0.008	0.078	0.040	0.008	0.108	0.004	1.58	0.133	
0.032	0.012	0.23	0.020	0.010	0.317	0.004	0.68	0.088	
0.013	0.029	0.51	0.094	0.008	0.100	0.010	1.08	0.198	Mean
2.6%	2.9%	25%	19%	16%	10%	10%	54%	99%	% recovery
65	94	79	65	28	97	55	29	109	Coefficient of variation

Table 37 - Organic acid level determinations on samples from bales of hay treated in the fourth field trial after six weeks storage.

* - Propionic acid extracted from hay by heating in kilner jars.

Determined organic acid levels * % on wet hay									
0.5% propionic acid	1.0% propionic acid	2.0% propionic acid	0.5% propionic acid:sorbic acid 9:1 (w/w)		1.0% propionic acid: sorbic acid 9:1 (w/w)		2.0% propionic acid: sorbic acid 9:1 (w/w)		Treatment
P.A	P.A	P.A	P.A	S.A	P.A.	S.A.	P.A.	S.A.	Acid Determined
0.064	0.05	0.12	0.072	0.008	0.041	0.007	0.75	0.050	Acid levels % by weight on the hay samples
0.019	0.07	0.07	0.035	0.010	0.175	0.010	0.84	0.029	
0.023	0.14	0.04	0.018	0.006	0.660	0.010	0.78	0.061	
0.045	0.44	0.01	0.011	0.006	0.042	0.010	0.40	0.054	
0.188	0.36	0.19	0.012	0.009	0.029	0.022	0.24	0.068	
0.138	0.48	1.33	0.047	0.008	0.051	0.007	1.02	0.034	
0.029	0.91	0.29	0.034	0.009	0.585	0.009	1.12	0.356	
0.328	2.07	0.97	0.266	0.010	0.040	0.006	1.10	0.095	
0.012	1.45	0.78	0.118	0.006	0.026	0.006	0.89	0.052	
0.032	0.17	1.42	0.068	0.005	0.010	0.005	0.80	0.036	
0.088	0.61	0.52	0.069	0.008	0.166	0.009	0.79	0.084	Mean
18%	61%	26%	14%	16%	17%	9%	40%	42%	% recovery
110	104	102	71	22	140	51	34	110	Coefficient of variation

Table 38 - Organic acid level determinations on samples from bales of hay treated in the fourth field trial - after three months storage.

* Propionic acids extracted from hay by heating in Kilner jars.

field trial, if the immediately after baling results are compared, which could be explained by the fact that a single large jet was used to apply the preservative in the fifth field trial whereas a multi-jet system was used in the fourth field trial. It would appear, therefore, that the multi-jet system applied the preservative more evenly than the single jet system.

The results in table 39 showing acid levels after five weeks storage, gave lower coefficients of variation than those from the hay before storage, which suggests that the distribution of acid may have improved during storage, probably due to diffusion, although this could only occur if the hay did not deteriorate.

Photographs one and two show hay which had been sprayed with fluorescent dyes, using hay preservative applicators, and which was then viewed under an ultra-violet lamp in a dark room. The hay in photograph one had been sprayed in the fourth field trial, at a rate of 1.0% with 0.1% primuline in a 0.2% polyvinyl alcohol solution, in water and the hay in photograph two had been treated, in the fifth field trial, at a rate of 2.0% with a 0.5% fire orange suspension in water.

The photographs show firstly the improved fluorescence of fire orange over primuline on hay and secondly the patches of dye occurring on the hay suggesting similar patches of preservative would occur, on hay, after application, this agreeing with results in tables 36 and 39.

Determined propionic acid levels * % on wet hay				
At baling		After 5 weeks storage		
2.0% propionic acid	2.0% propionic acid: water (1:1 v/v)	2.0% propionic acid	2.0% propionic acid: water (1:1 v/v)	Treatment
0.18	0.01	0.23	0.06	Acid levels % by weight on the hay samples
2.89	0.13	0.13	0.01	
0.41	1.92	0.49	0.03	
1.31	0.11	1.27	0.33	
0.95	0.02	1.06	0.80	
0.17	0.37	0.44	0.15	
0.44	1.15	0.44	0.04	
0.12	1.94	0.24	0.53	
0.53	0.17	0.68	0.32	
0.09	0.04	0.14	0.11	
0.71	0.63	0.94	0.23	Mean
36%	63%	47%	23%	% recovery
115	117	72	107	Coefficient of variation

Table 39 - propionic acid level determinations on samples from bales of hay treated in the fifth field trial, immediately after baling and after five weeks storage.

* - propionic acid extracted from hay by heating in kilner jars.

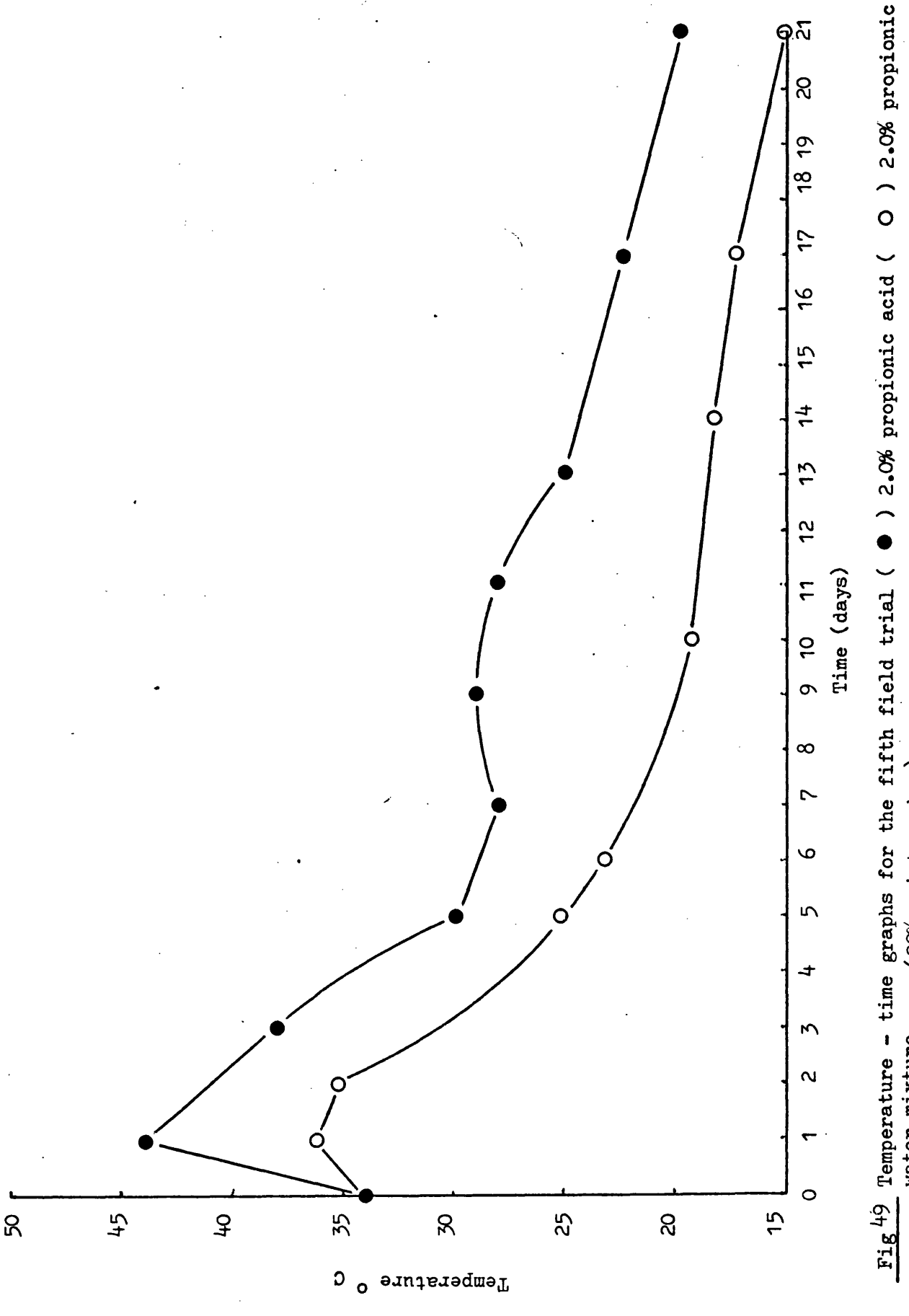
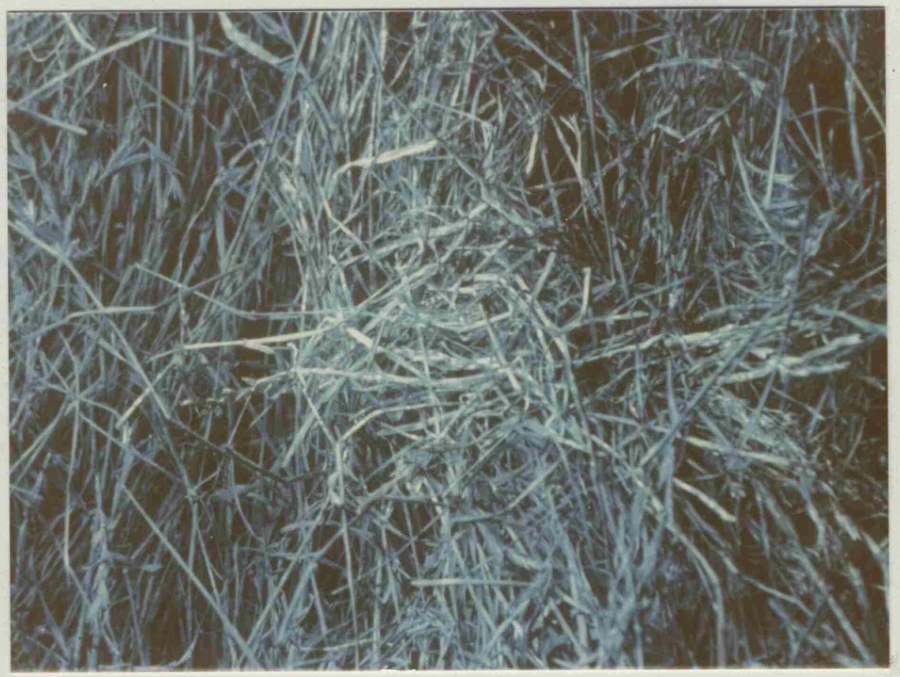


Fig 49 Temperature - time graphs for the fifth field trial (●) 2.0% propionic acid (O) 2.0% propionic acid with water mixture. (22% moisture hay)



Photograph 1 - Hay sprayed at a rate of 1.0% with 0.1% primuline in a 0.2% polyvinyl alcohol solution in water, using a multi-jet hay preservative applicator.

Photograph 2 - Hay sprayed at a rate of 2.0% with a 0.5% fire orange suspension in water, using a single-jet hay preservative applicator.

APPENDIX

The distribution of propionic acid on silage using two different silage preservative applicators

Although not directly related to the problems of preservative application to hay, the distribution of propionic acid on silage was compared using two different silage preservative applicators, the same method of acid level determination being used as with hay.

The propionic acid was extracted from the grass by soaking in 0.6N H_2SO_4 for seven days.

Two experiments were carried out to assess the distribution of propionic acid obtained, using firstly, a Feed Services silage preservative applicator which sprayed the acid onto the grass using an electric compressor and secondly a British Petroleum (B.P.) applicator which relied upon gravity to apply the acid. With both applicators the acid was sprayed onto the grass immediately after cutting, using a single jet system.

For each treatment, ten random samples were taken from the grass in the collection trailer immediately after spraying, and the propionic acid levels were determined as previously described. The results are summarised in tables 40 and 41.

Considering the results for the first experiment (Table 40), a comparison of the coefficient of variations suggests that the Feed Services applicator, applying acid at a rate of 0.2%, gave a more even acid distribution than the B.P. applicator applying acid at a rate of 0.5%.

In the second experiment the procedure was repeated except that different application rates were used. The results (Table 41) suggest that the Feed Services applicator gave a more even acid distribution than the B.P. applicator at rates of 0.1%, however, at the higher application rates of 0.25% and 0.375% the B.P. applicator gave a similar distribution of acid on the grass, to the Feed Services applicator.

	Determined propionic acid levels % on wet grass	
	B.P. applicator at a rate of 0.5%	Feed Service applicator - at a rate of 0.2%
	0.10	0.09
	0.11	0.10
	0.38	0.19
	0.14	0.12
	0.20	0.14
	0.26	0.09
	0.16	0.10
	0.27	0.20
	0.12	0.07
	0.10	0.11
Mean	0.184	0.12
% Recovery	37	60
Coefficient of Variation	43	14

Table 40 - Determinations of propionic acid levels on grass sprayed with propionic acid using a B.P. applicator and a Feed Services applicator.

Determined propionic acid levels % on wet grass						
B.P. applicator			Feed Services applicator			Applicator
0.1%	0.25%	0.375%	0.1%	0.25%	0.375%	rates
0.002	0.20	0.28	0.051	0.062	0.23	
0.002	0.33	0.24	0.082	0.170	0.22	Acid
0.005	0.13	0.35	0.096	0.150	0.13	levels
0.013	0.26	0.22	0.036	0.084	0.20	% by
0.001	0.20	0.36	0.045	0.035	0.38	weight
0.016	0.19	0.15	0.101	0.075	0.19	on
0.009	0.27	0.26	0.113	0.340	0.29	grass
0.005	0.21	0.14	0.106	0.210	0.21	samples
0.011	0.13	0.30	0.087	0.230	0.19	
0.004	0.13	0.36	0.071	0.130	0.38	
0.007	0.20	0.27	0.079	0.150	0.24	Mean
7%	80%	72%	79%	60%	66%	% recovery
63	31	28	36	60	33	Coefficient of Variation

Table 41 - Determination of propionic acid levels on grass sprayed with various levels of propionic acid using a B.P. applicator and a Feed Services applicator.

These results shown in tables 40 and 41 agree with those obtained using hay preservative applicators in that firstly, the preservatives were applied unevenly and secondly, this poor distribution was not improved by increasing the application rates.

The results in Table 42 show a visual assessment of the fluorescence of the fluorescent dye tracer fire orange which had been sprayed onto grass using the B.P. and Feed Services applicators at various rates, a 0.5% suspension of fire orange in water being used.

Two independent observers (A and B) made the recordings when viewing the grass samples in a dark room under a 125 watt ultra-violet lamp. Two recordings were made for each sample, one side being assessed, the sample then being turned over and the second side being assessed.

A scale of 1 to 10 was used to record the fluorescence, the higher the number the stronger the observed fluorescence. This arbitary scale was based on one of the samples which had been sprayed at a rate of 0.25% using the Feed Services applicator. When this sample was viewed under the ultra-violet light it was given a rating of 5 and then all the remaining samples were compared with this standard sample and assessed accordingly.

Samples treated with the B.P. applicator at rates of 0.375% were not obtained because the supply of grass became exhausted.

These results suggest that a more even distribution of the dye on the grass was obtained, by using the Feed Services applicator at rates of 0.1% and 0.25% than by using the B.P. applicator at the same rates.

Visual assessment of fluorescence *												
B.P. Applicator						Feed Services applicator						Applicator
0.1%		0.25%		0.375%		0.1%		0.25%		0.375%		Rate
A	B	A	B	A	B	A	B	A	B	A	B	Observer
T T	T T	0 T	0 0			3 3	3 3	Standard		5 5	5 4	
T 1	1 1	0 T	0 0			2 2	2 3	6 5	5 5	7 5	6 5	
T T	T T	T T	T 0			7 5	7 5	4 5	3 5	5 5	4 5	
T T	T T	0 0	0 0			4 2	5 3	5 6	5 6	3 4	4 5	
T T	0 0	T 1	T T			5 6	5 6	6 6	6 6	6 7	5 6	
1 T	2 T	T T	T T			6 5	6 5	3 2	6 2	6 6	5 6	
						3 4	3 4	4 5	5 5	6 6	6 5	
						5 5	5 6	3 5	5 6	4 3	3 3	
						4 4	4 4	5 5	5 6	7 6	7 6	
						5 4	6 4	6 5	7 5	3 4	3 5	

Table 42 - Visual assessment of the fluorescence of the dye fire orange on grass samples which had been sprayed with a 0.5% fire orange suspension in water, at various rates using a B.P. applicator and a Feed Services applicator.

* - T - a trace of fluorescent dye observed

0 - No fluorescent dye observed.

DISCUSSION

SECTION A - ESTIMATION OF HAY DETERIORATION

In order to assess the effectiveness of a preservative treatment upon hay, a parameter was needed to determine the extent of the deterioration that had taken place within the hay after treatment. The deterioration of hay is almost entirely due to microbial activity, therefore an examination of the microbial population in the hay before and after storage would appear likely to give a good estimate of the extent of deterioration and consequently microbiological counting techniques were employed extensively in this work.

a) Microbiological Counting

When trying to estimate the microbial numbers in hay, problems arise due to its inhomogenous nature. The hay in a bale consists of the stems, leaves and flowers of not only grass but also of many weeds including docks, dandelions, buttercups and clover. These various constituents result in the growth requirements for micro-organisms including water activity, available nutrients, minerals and p.H being unevenly distributed through the bale and therefore microbial growth and sporulation will tend to occur in patches. Consequently, when trying to estimate the microbial numbers in large quantities of hay, for example a bale, the estimated counts obtained by analysing one sub-sample may be very different from the estimated counts obtained by analysing another sub-sample from the same bale. This has been shown from results in this work where two samples from the sample bale have been known to give counts 100 fold different.

The larger the samples of hay that can be analysed, the less

significant this problem becomes, however, if too large, the sample becomes impractical to handle and therefore it was considered that samples between 5g and 10g dry weight would be sufficiently large for microbial analysis. Also the results in fig 8 show that samples larger than 10g create the further problem of poor spore removal if the air sampling technique is employed.

Replication of samples is one method for obtaining more representative results, however, when using microbial counts to compare the microbiological condition of two or more hay samples, which was frequently considered necessary in this work to compare the effectiveness of various preservatives at preventing the deterioration of hay, it becomes difficult to distinguish between the variation amongst the replicate samples from the same batch of hay and the variation due to the effect of preservatives in preventing microbial growth and sponulation. This point is well demonstrated if the microbial counts for most of the dewar flask experiments and field trials are examined.

In an attempt to overcome this problem, statistical analysis was frequently employed for microbiological counts and this showed that the variation between replicate samples was sometimes considerable and that large differences in the counts for two separate batches of hay were not always found to be significant ($P < .05$). For example, in the sixth dewar flask experiment (Table 27) considering the fungi incubated at 45°C, the untreated hay had a count of 8×10^4 spores /g dry hay after storage, whereas the 0.75% propionic acid treated hay had a count of 299×10^4 spores /g dry hay after storage and yet analysis showed that there was no significant difference between these two spore counts.

Despite these disadvantages microbial counts for assessing the extent of deterioration of hay were used extensively in this work because firstly other measurements including p.H and glucosamine and diaminopimelic acid level determinations suffered from the same sampling errors, leaving no alternatives, except, possibly following temperature changes, which is discussed later and secondly by incubating a proportion of the agar plates at high temperatures the number of spores present which were potentially hazardous to man and animals, could be estimated.

A further advantage in using microbial counts is that when a preservative has been added to hay, any selectivity for a particular group of micro-organisms can only be detected by this method. For example, an organic acid may fail to preserve hay because acid resistant micro-organisms will grow and degrade the preservative which then allows the remaining microflora to multiply. This theory was suggested by results from the tenth dewar flask experiment (fig 39) where acid resistance in bacteria, from hay treated with 1.0% of a propionic acid: sorbic acid mixture, increased during storage. If this was the case then the numbers of acid resistant micro-organisms would initially increase in proportion to the remaining microflora and this could only be shown by counting viable micro-organisms.

In this work two methods were regularly used for assessing the microbial numbers in hay, namely a washing-serial dilution - pour plate technique and an air sampling technique.

It was considered that the dilution plate technique gave a more representative estimation of the microbial population of the hay samples because the micro-organisms were removed from the hay more effectively than with the agitation method used in the air sampling technique.

However, there were two problems encountered when using the dilution plate technique.

Firstly, many of the spores transferred from the hay to the water did not wet and therefore tended to float on the surface in clumps. This caused problems when trying to serially dilute the solutions because these spores tended to adhere to the outside of the pipette. In an attempt to overcome this problem the wetting agents Teepol and Tween 80 were added to the washing water. They were expected to increase the estimated microbial counts of hay because when the spore suspensions were viewed microscopically, the wetting agents had reduced the formation of clumps resulting in a more even spore suspension. However, the results in table 6 show that the wetting agents had not increased the estimated microbial count, probably because there were two factors working in opposition. Firstly, when no wetting agent was added the spores floated, therefore during serial dilution, some were carried over on the tip of the pipette which would give high estimated counts, however, when the wetting agents were added there was less spore clumping which would also give higher spore counts because a clump of spores would probably only form one colony on the agar plate.

The second problem was that bacteria and actinomycetes were very difficult to separate by the use of selective media. When sampling deteriorated hay, where the actinomycete population was large, their colonies in the agar plates produced antibiotics which severely inhibited the developement of bacterial colonies. When sampling clean hay, where the actinomycete population was small, a low dilution of the hay washings was needed to obtain a countable number of colonies on the plates, however, the bacterial colony numbers were usually very high at these dilutions and they tended to overgrow the actinomycete colonies.

In an attempt to overcome these problems and to simplify the microbial estimations of hay the air sampling technique previously described was devised, however, this method also had its problems.

Firstly, when the spore suspensions in the container were drawn through a cascade impactor air sampler, microscopic examination of the plates showed that a considerable proportion of the spores were present in chains and clumps and many were adhering to debris from the hay including pollen grains, mite skins and particles of grass stems, leaves and flowers. In order to remove this debris an agar plate was placed in the top section of the Anderson air sampler during sampling, however, microscopic examination of the surface of this plate revealed the presence of large numbers of spore chains and clumps which would never have reached the plates used for estimating microbial spore numbers.

This point could, at least in part, explain why the estimated microbial spore counts of hay were approximately 100 fold less when using the Anderson air sampler than when using the cascade impactor sampler (Table 8) (Gregory and Lacey 1963a). Another reason was that the cascade impactor did not distinguish between non-viable and viable spores whereas the Anderson sampler only estimated the number of spores that were capable of growing on the agar media employed. This apparent loss in the viability of many spores could be due to one or more of several reasons. Firstly a natural loss due to ageing, secondly, by the activity of sporicidal compounds produced by other micro-organisms in the hay, thirdly, some mesophilic spores could possibly have been killed due to a pasteurisation effect in hay that had heated and fourthly, where hays had been treated with a preservative it is to be

expected that a proportion of the spores will have been killed especially if the hay had also heated.

The second problem is shown by figs 9a and 9b, where the spore concentration, after agitation, decreased inside the container and that this apparent loss was increased if the fan was in operation. It was originally thought that this loss was mainly due to leakage between the container rim and the lid, however, the sealing of this gap using double sided adhesive tape made no difference and it was also noticed that many spores were adhering to the aluminium foil lining the apparatus. Therefore, it was concluded that electrostatic attraction between the spores and the foil accounted for this spore loss.

A third problem encountered with the air sampling technique was that after the hay samples had been agitated inside the container a considerable number of spores remained on the hay, as was revealed by microscopic examination. Assuming that the washing method removed the majority of spores from the hay, then a comparison of estimated microbial counts by this method and by the air sampling technique could give an estimate of the number of spores removed when the hay was agitated inside the container.

The results of such a comparison (table 8) suggest that approximately 1% of the spores were removed, however, this figure does not allow for the considerable errors in both techniques which have previously been outlined.

It would appear, therefore, that the air sampling technique would be improved if the hay could be thoroughly homogenised in the air inside the container, in order to remove the majority of spores from the

hay samples and to break up the spore chains and clumps.

The results in fig 7 show that drying the hay before sampling greatly enhanced the removal of spores from the material, presumably because water created cohesive forces between the spores and the hay.

b) The estimation of the microbial population of hay by determination of chitin and diaminopimelic acid (D.A.P.A.) levels

Microbial counts could only be reliably used to differentiate between good hay, mouldy hay and hay likely to cause Farmer's lung, therefore it was considered that the estimation of the chitin and D.A.P.A. levels in hay could give a more rapid and convenient estimate of microbial growth in hay, without losing any accuracy.

i) Chitin estimations

Chitin was estimated by acid hydrolysis to glucosamine which was then determined colourimetrically.

The results in fig 10a and 10b show that the colourimetric determination of glucosamine, which had been added to a hay hydrolysate, by the Tsuji method, suffered from less interference by other hay hydrolysis products, than the Morgan-Elson method and the error due to this interference was considered insignificant in comparison with errors due to sampling and variations in the chitin content of different fungal species. Consequently the Tsuji method was employed in this research, in preference to the less convenient method described by Philips (1974), where interfering compounds including neutral sugars and amino acids had to be separated from the glucosamine by an ion-exchange column before

the glucosamine was estimated by the Morgan-Elson method.

The results in fig 11 show a direct relationship between the fungal spore counts and the estimated glucosamine level of hay, the levels estimated by the Morgan-Elson method being lower than when the Tsuji method was employed, presumably because the former underestimates the glucosamine present (fig 10a).

Considering the results obtained by the Tsuji method (fig 11), a deteriorated hay having a 1000 times greater spore count than a clean hay, contained only three times the quantity of glucosamine. Philips (1974) showed that the glucosamine content of an Absidia spp was 6.7% for the spores and 12 -18% for the mycelium, however, when this fungus sporulated there was no appreciable decrease in the glucosamine content which indicated that the mass of the spores was small in relation to the mass of the mycelium. Therefore considering a fungus growing in hay, when it sporulates, it considerably increases the spore count but has little effect on the glucosamine level in the hay.

Table 10 shows a four fold variation in the glucosamine content of fungi which commonly grow in hay, which is similar to results, obtained by Philips (1974) for storage fungi from grain. It would therefore appear that in order to be able to relate the glucosamine content of hay to the mass of fungal mycelium present it would be necessary to know the proportion of individual fungi present, which could only be determined by viable counts which would overestimate heavily sporulating species.

These variations in the glucosamine contents of different fungal species are important when considering the fungal succession that occurs

in hay (Gregory et al 1963b, Dewar flask experiment 10). Phycomycete species usually appear first and table 10 shows that they have a high glucosamine content when compared with fungi that become dominant at later stages of deterioration, therefore the ratio between the estimated glucosamine content of hay and the fungal mycelial weight could alter with the stage of deterioration. This variation would be further exaggerated by the fact that nutrient availability also affects the glucosamine content of fungi (Philips 1974) and that dead aswell as viable chitin is estimated by this method, and the content of non-viable mycelium in hay will probably increase during storage.

Golubchuck et al (1960) tried to relate the chitin content of wheat to other parameters of microbial activity including seed viability, fat acidity and dilution plate counts, however they obtained no correlation between any of these parameters.

They noted that the chitin levels of mouldy wheat grain varied between 0.05% and 0.1%, however, the levels of glucosamine on hay recorded in the dewar flask experiments usually varied between 1.0% and 4.0% suggesting that hay supports a higher level of fungal growth than wheat grain.

ii) D.A.P.A. estimations

Most of the information relevant to estimating the fungal growth in hay by glucosamine level determinations, applies to estimating bacterial and actinomycete growth by D.A.P.A. level determinations, although there is less information available.

Tables 11a and 11b show a ten fold variation in the percentage dry

weight of cells constituted by D.A.P.A. amongst the bacteria and actinomycetes examined.

Ion exchange columns were used to purify the D.A.P.A. in the hay hydrolysate, although the calculated levels did not appear to be any more consistant with the microbial counts and heating, than the glucosamine levels.

Relating D.A.P.A. levels to bacterial number in hay could involve less error because there is not the problem of sporulation, however difficulties arise due to errors of bacterial estimation in hay as previously discussed and also some means of distinguishing between bacteria and actinomycetes is needed.

It appears the main disadvantages of using these determinations of microbial chemical components for estimating microbial numbers are, firstly that both non-viable and viable material is estimated, whereas only the viable material is of interest. Secondly the presence of potentially hazardous micro-organisms cannot be detected, and thirdly with a mixed microbial population as occurs in hay, variation between these constituent levels, as a percentage dry weight of the micro-organisms, occurs depending on the species, the stage of growth and the nutrients available.

The advantages are that firstly, as opposed to microbial counts, the weight of vegetative growth is estimated which is more important when considering the quantity of microbial activity that has occurred, as this will relate to the extent of hay degradation and the production of microbial toxins. Secondly they are relatively rapid and thirdly there are no problems with removing the micro-organisms from the hay.

These estimations probably could be used to distinguish between good hay and deteriorated hay and the results from the dewar flask experiments support this conclusion, however, considerably more work needs to be done, and there is always the problem of sampling errors.

c) Temperature changes

Having established that the use of microbial counts to estimate the extent of hay deterioration, leaves much to be desired, what alternatives present themselves?

Probably the most accurate method is to follow the temperature changes, and it appears safe to assume that if hay has not heated during storage then there has been little if any, microbial activity (Gregory et al 1963b).

The main advantage of using heat production to estimate microbial activity is that although in a bale of hay the heat is probably produced unevenly due to the inhomogenous nature of hay, some of this heat would be transferred from the hotter areas to the cooler regions by heat conduction and gas and water vapour movement (Currie and Festenstein 1971) and therefore would be spread more evenly throughout the bale. Consequently, by measuring the temperature of one part of the inside of a bale, the average temperature of the whole bale can be estimated and the error due to the inhomogenous nature of hay would be considerably reduced.

The measurement of the maximum temperature attained by hay was considered to be an unsatisfactory method for estimating the quantity of heat produced, and hence the microbial activity, because different hays produced different heating patterns. This became more evident when

preservatives had been added to hay, because they often flattened out the temperature time curve where although the maximum temperature had been reduced, the quantity of heat produced expressed by the area under the temperature -time curve was similar and sometimes greater because the hay had heated over a longer period of time. Examples of this can be seen in the 4th and 7th dewar flask experiments (figs 28 and 31). Therefore the measurement of heating by degree days was adopted which was an expression of the area under a temperature - time graph and was related to the quantity of heat produced in the hay and therefore related to the extent of plant and microbial respiration. This relationship is not direct, however, because the specific heat of hay changes during deterioration (Currie and Festenstein 1971) and the rate of heat loss will vary with the temperature of the hay.

The relationship between heating, as measured in degree days, and final microbial counts varies with different hays. A direct relationship is suggested in the fourth field trial (table 35) where generally in the hays where considerable heating had occurred the final microbial counts were higher than in hays where less heating had occurred, this being most obvious if the thermophilic actinomycetes are considered. However, both the 42% moisture hay in the first field trial (table 32) and the 44% moisture hay in the third field trial (table 34) heated considerably and yet the final total microbial counts were higher in the third field trial (35×10^6/g hay) than in the first (1.5×10^6/g hay). Also in the hay in the second field trial less heating occurred (table 33) than in first field trial however the final microbial spore counts were similar (5.0×10^6/g hay).

These inconsistent results could be due to three possible reasons.

Firstly, variation in the initial microbial inoculum, for example, if the 42% moisture hay in the first field trial is compared with the 19% moisture hay in the second field trial the microbial counts at baling are higher for the latter hay, suggesting that a large initial inoculum leads to a high final count.

Secondly, the readily available nutrient content of the hay. For example, by comparing the first and third field trials, the latter hay was greener than the former suggesting it contained a higher nutrient content, therefore more microbial activity occurred resulting in higher spore counts.

Thirdly, the bale compaction. Currie and Festenstein (1971) demonstrated the importance of aeration for microbial activity in hay and because it was impossible to ensure equal compaction of the hay for all the field trials, the aeration of the hay during storage may have varied between field trials. Also as baled hay deteriorates there is a loss of compaction.

It appears therefore, that certain qualities of hay, including readily available nutrient content, initial microbial inoculum, aeration during storage and moisture content are likely to affect the relationship between heat production and microbial activity within the hay.

There appears to be one disadvantage in using temperature measurements to estimate the microbial activity in hay, in that it is often difficult to distinguish between plant respirational heat and microbial respirational heat, because these heating phases usually merge together.

Ideally a preservative is required to prevent both forms of respiration because they both cause a loss in the nutritional value of hay (Gregory et al 1963b Festenstein 1966), although microbial activity is the more

important.

Complete prevention of heating in hay by chemical treatments can be seen in the second and sixth dewar flask experiments (fig 26 and 30) and in the fourth field trial (fig 48) where the 2.0% treatment with the propionic acid: sorbic acid mixture preserved the hay.

With hay at low moisture levels (20%) some plant respiration appeared to occur without microbial activity as can be seen in the temperature - time graph for the fifth field trial (fig 49), where the maximum temperature was attained after 24 hours. This heating was considered to be due to plant enzymes primarily because microbial heat output usually reaches its maximum after several days storage and also propionic acid, which usually delays the maximum temperature, had been applied to this hay. Gregory et al (1963b) noted that damp hay usually showed double heating peaks the first one occurring after only 1 to 2 days and the second one a few days later. They concluded that the first heating peak was due to plant enzyme activity and the second to microbial activity.

It was concluded that during this investigation when testing the effectiveness of a preservative on hay, temperature changes should be followed and the microbial population before and after storage should be assessed as accurately as possible.

SECTION B - THE SCREENING OF ANTI-MICROBIAL COMPOUNDS

a) Using chemically defined agar media

The objective of these experiments was to provide a rapid assessment of the anti-microbial characteristics of chemical preservatives, including the effect of p.H, and their relative activity against particular groups of micro-organisms. This was done for two reasons.

Firstly, only chemicals with a strong anti-microbial activity against the majority of hay micro-organisms would be likely to be effective hay preservatives.

Secondly, by comparing the properties of different compounds, it may have been possible to formulate mixtures whose components acted synergistically. Propyl hydroxybenzoate had a stronger anti-microbial activity at low p.H values (table 21) therefore it would probably be more effective if applied to hay as a solution in an acid, for example propionic acid, whereas glutaraldehyde had stronger activity at high p.H values therefore it would probably be a more effective preservative if mixed with an alkaline solution before being applied to hay.

The results in tables 15,16 17 and 18 show that the organic acids used, had anti-microbial activity against all the micro-organisms tested, and suggest that at p.H values between 5.0 and 6.0 (p.H of moist hay at baling) the n-butyric acid: sorbic acid mixture and the n-butyric acid: propionic acid: sorbic acid mixture would make the most effective preservatives. However, n-butyric acid was later considered impractical due to its expense and unpleasant smell therefore the propionic acid:

sorbic acid mixture appeared to have the best potential.

The effect of a decrease in p.H increasing the activity of these organic acids can clearly be seen, although it is slightly less pronounced with the fungi probably because most fungal species prefer slightly acidic conditions for growth, whereas bacteria and actinomycetes especially the latter group, prefer neutral to weakly alkaline conditions and it was noticed that on the control media at p.H 5.0, many of the actinomycete species, especially the thermophiles, grew weakly. Table 19 shows that when added to the buffers used in the preparation of the agar media, the organic acids lowered the p.H and therefore in the results at p.H 5.0 (table 15) the inhibition of the actinomycetes and bacteria could have been a p.H effect aswell as an inhibitory effect of the acids. This could possibly explain why sorbic acid was less effective against the actinomycetes than the volatile fatty acids, as it is a weaker acid.

Lactobacilli spp were found to be more resistant to organic acids than most aerobic micro-organisms which agrees with the results of Woolford (1975a), and the medium of Keddie (1951) incorporates an acetate buffer at p.H 5.4 as part of its selective properties for Lactobacilli. It is therefore interesting to note that, in the first five dewar flask experiments, although Lactobacilli did increase in numbers on some hays, this increase in proportion to increase in the other microbial species, was not affected by organic acid treatments.

The work of Pepys et al (1963) showed that for fresh hay to support the growth of thermophilic actinomycetes it initially required either microbial growth or infiltration with ammonia to raise its p.H value. Therefore if a preservative could prevent bacterial and fungal growth,

the latter being more important at lower moisture levels, then the thermophilic actinomycete species and probably most other actinomycetes would be inhibited.

The p.H of hay at baling is usually between 5.0 and 6.0, therefore the results in tables 15 and 16, showing the inhibitory levels of the organic acids in agar media at p.Hs 5.0 and 6.0, are probably the most applicable to hay preservation. Considering table 16, 0.4% propionic acid prevented the growth of all the fungal species studied, 0.2% prevented the growth of all the bacterial species except for the Lactobacilli where 0.8% propionic acid was necessary. The corresponding levels for the propionic acid: sorbic acid mixture (9:1 w/w) are 0.2%, 0.2% and 0.8% and for the n-butyric acid: propionic acid: sorbic acid mixture (45:45:10 w/w/w) are 0.2%, 0.2% and 0.8%. Therefore it appears that none of the mixtures examined were significantly more effective than propionic acid alone, and when considering them for hay preservation, these small differences in activity are insignificant when compared with other factors affecting the chemical preservation of hay, which are discussed later.

There are several inaccuracies in using chemically defined agar media for screening compounds as hay preservatives. Firstly, no allowance is made for chemical reactions between the preservatives and components of the hay. Secondly chemicals dissolved in agar media are in contact with only part of the micro-organisms and are in a dilute form, these conditions not being similar to those where the chemicals are sprayed in an undiluted form, over the micro-organisms. Thirdly different aeration conditions exist in a bale of hay and in agar plates and these in turn, will affect both the activity of many preservatives and the

growth of the micro-organisms. Fourthly, agar media and hay will have different water activities which will also affect preservative and microbial activity.

b) Using hay stored in dewar flasks

The dewar flask experiments were designed to study the comparative preservative activities on hay at different moisture levels of various chemical treatments, and to try and determine why certain preservatives were more effective than others, this work concentrating on the organic acids.

Also by looking for increases in particular groups of micro-organisms it was hoped to determine which, if any, were responsible for the degradation of preservatives.

Finally it was hoped to be able to correlate results from these experiments with results obtained in the field trials so that information from hay stored in dewar flasks would be used to predict what would happen in baled hay.

The abilities, of various chemical mixtures, to preserve hay stored in dewar flasks were studied.

The conclusion from these experiments was that because they prevented heating more efficiently than other treatments, the volatile fatty acids were considered the most effective hay preservatives and for further reasons, including lack of obnoxious smell and cost, propionic acid was considered the most practical.

The addition of sorbic acid to the volatile fatty acids had a dubious beneficial value. In experiment two (fig 26) sorbic acid appeared to enhance the activity of Hay Shield and in experiment six (fig 30) the 0.75% treatment with the propionic acid: sorbic acid mixture prevented heating of the hay for 66 days whereas the hay treated with 0.75% propionic acid alone heated to 48°C. However, in experiment seven (fig 31) hay treated with 1.5% of the propionic acid: sorbic acid mixture heated a little whereas hay treated with 1.5% propionic acid alone did not heat during the 66 days storage.

These results, and those obtained using agar media, suggest the addition of sorbic acid to volatile fatty acids is equivocal.

Formaldehyde and glutaraldehyde both appeared ineffective on hay, although they both had strong anti-microbial activity in chemically defined agar media (table 21). The facts that glutaraldehyde was more inhibitory at higher p.H values and that fresh hay is weakly acidic suggest glutaraldehyde may be improved as a hay preservative if used in conjunction with a strong alkali, however, because not even a 1.5% treatment had any significant effect in controlling the heating of hay (fig 32), glutaraldehyde was not considered to have potential as a hay preservative.

In experiment 9 (table 30 fig 33) paraformaldehyde applied at 1.5% did not control heating, however, 1.5% of a paraformaldehyde:sodium nitrite mixture controlled heating of hay, suggesting a synergistic activity although the use of sodium nitrite as an animal feed preservative is dubious because of its possible mammalian toxicity and damaging effect on the rumen flora.

A possible explanation for the apparent inactivation of glutaraldehyde and formaldehyde when applied to hay is that, being very reactive compounds, they chemically combined with components of the hay including proteins and sugars, although in the case of formaldehyde the organic acids it was mixed with, may have also been partly responsible. Paraformaldehyde would have slowly released formaldehyde in the hay, however, the levels of formaldehyde were probably never sufficiently high to control microbial activity in hay, although microbial increases were prevented in poultry litter by 1% and 3% paraformaldehyde treatments (Veloso et al 1974).

The propionic acid: propyl hydroxybenzoate mixture applied at 1.0% to hay delayed heating for longer than the propionic acid: sorbic acid mixture at 1.0% (fig 33), however, when the former hay did heat more heat was produced. From table 21 there appeared to be no significant difference between the activity of these two mixtures, therefore as with sorbic acid, the beneficial value of adding propyl hydroxybenzoate to volatile fatty acids is dubious.

i) The changes in deteriorating organic acid treated hay

From the results in dewar flask experiments 1 - 10, there are a few clues as to the changes that occur in damp hay treated with organic acids when it deteriorates in dewar flasks.

After the treated hay is packed into the flasks there is initially no activity, unless the grass is very fresh and moist and the acid level is low, in which case some grass and microbial respiration will occur, utilising oxygen, giving off carbon dioxide and causing the

temperature to rise.

Assuming sufficient organic acid has been applied to prevent this activity, there are no significant changes for the first few days of storage, as can be seen from the temperature - time graphs for most of the organic acid treated hays in these experiments. During this period the acid levels remain constant (fig 40). However, at acid levels used in these experiments (0.25% to 2.0%) there will be slow growth of organic acid resistant micro-organisms in the hay especially in the areas with lower levels of acid, although this activity is not sufficient to cause detectable heating. Gradually the numbers of acid resistant micro-organisms, which are probably mainly bacteria (fig 41), will slowly increase depleting oxygen and producing carbon dioxide (fig 38), their growth being permitted by the strong selective pressure from the organic acids. It is also possible that some of these micro-organisms are members of the hay microflora that have acquired acid resistance.

As these micro-organisms grow, some will start to degrade the organic acids possibly by using metabolic pathways described in the introduction section of this thesis and gradually the organic acid levels will decrease. As they do so, further species of the microflora will not be inhibited and will therefore grow causing the acid levels to decrease more rapidly. Consequently, within a short period of time there is rapid microbial growth consisting firstly of mesophilic micro-organisms but as the temperature reaches 40°C, the mesophiles will stop growing and the thermophiles will start to grow, with thermophilic bacteria and actinomycetes taking the temperature to above 60°C if the conditions are adequate, (Gregory et al 1963 Festenstein et al 1965).

The initial phase of this heating is probably mainly caused by fungi as hay is weakly acidic. However, as their activity raises the hay p.H, actinomycetes start to grow (Pepys et al 1963).

The higher the level of organic acid the slower the initial rise in temperature, as shown in dewar experiment three (fig 27), presumably because more time is required to reduce the acid to sub-inhibitory levels.

The hay in these experiments was very unlikely to heat above 70°C because Currie and Festenstein (1971) showed that for hay to heat to ignition in dewar flasks, air, initially with a high relative humidity and finally with a lower relative humidity, had to be passed through the hay and also, their dewar flasks were stored in ovens where the surrounding air was heated as the hay temperature increased. Considering the dewar flask experiments in this work, there was insufficient aeration, too much heat loss through the top and the hay remained too damp for self-ignition to occur.

The temperature of the hay remains high for a few days, due to considerable microbial activity, but then decreases for one or more of several possible reasons, these being lack of oxygen, loss of water due to evaporation, accumulation of carbon dioxide and toxin production from some microbial species, for example antibiotics. However, probably the most important factor is that the readily available nutrients are depleted, therefore microbial activity, and consequently the temperature, decreases.

The utilisation of readily available nutrients creates a selective

pressure within the hay which permits the growth of micro-organisms capable of producing enzymes which can degrade the larger polymers including cellulose, hemi-cellulose and proteins. The activity of these enzymes will then produce further nutrients.

If the temperature has dropped sufficiently mesophilic micro-organisms will again grow increasing the temperature until it is suitable for the thermophiles. The conditions in the hay are now, however, different than at the beginning of the first heating phase, due mainly to the many microbial metabolic products present, probably the most important of these being volatile nitrogen compounds derived from protein breakdown, which have raised the p.H of the hay so that it is now weakly alkaline (fig 22). These conditions will favour the growth of actinomycetes and it is possible that these micro-organisms grow mainly during this second heating phase and the fungi mainly during the first heating phase, although there is no evidence to support this possibility in figs 35 and 36.

In some hays it appears these two heating phases have merged together as in the glutaraldehyde treated hay in experiment eight (fig 32b), where there are small double heating peaks at the top of one large peak and it appears two large heating peaks had almost completely merged into one. In this experiment a fresh green hay was used, which probably contained sufficient nutrients to allow considerable microbial activity until further nutrients, from polymer breakdown, were produced.

Eventually for one or more of three possible reasons microbial growth and heating stop. These reasons are firstly, complete exhaustion of all possible nutrients although this seems unlikely

because hay that has heated contains some nutrient value (Benham et al 1975). Secondly, loss of water, possibly important in baled hay as the moisture level decreases during storage (table 35) but not in dewar flask hay because the moisture levels tend to increase. Thirdly, and probably the most important factor is the build up of anti-microbial metabolic products including volatile nitrogen compounds, antibiotics and organic acids including lactic, acetic, propionic and n-butyric which have appeared on gas liquid chromatogram traces of extracts from deteriorated hay.

Not all the results observed in these dewar flask experiments can be explained by this account. For example, why did some organic acid treatments completely prevent heating in hay (fig 26)? It could have been that these hays would have heated eventually if acid resistant micro-organisms had been allowed sufficient time to grow and reduce the acid levels.

In experiment six, the untreated hay showed three separate heating phases (fig 30). The third phase possibly could be explained by a diffusion out of the hay of compounds, probably volatile nitrogen compounds, which had built up during the second heating phase and prevented a continuation of microbial activity. Consequently as the levels of these compounds decreased micro-organisms were permitted to grow.

In experiment seven the hay, treated with 1.5% of the propionic acid: sorbic acid mixture, started to heat at day 52, but it then stopped almost immediately (fig 31). This is difficult to explain but it may have been caused by an extended, low microbial activity phase, due to the high organic acid level present, and if this hay

had been stored for longer eventually the temperature may have increased.

Another result difficult to explain is why neither of the 1.0% organic acid treated hays in experiment seven (fig 31) heated above 35°C. All that can be positively concluded is that it was not due to a lack of nutrients as the untreated hay heated to 65°C and neither was it because the organic acids remained at inhibitory levels as they had disappeared after 66 days storage (table 28).

iii) The use of results from hay stored in dewar flasks to predict changes in baled hay

An important question that needs to be answered is can these results from hay stored in dewar flasks be applied to hay stored in bales?

There seems little doubt that caution is needed when using these results to determine changes in baled hay because there are several important differences between the two situations. These differences include oxygen, carbon dioxide and water vapour diffusion rates, which are affected by variations in hay compaction and the impermeability of the dewar flask walls and are important factors affecting the heating of hay (Currie and Festenstein 1971). Further important considerations are heat insulation differences, and the fact that dewar flask hay was re-wetted whereas baled hay, unless it had been lying in the field and rained on, contained mainly sap moisture. Considering the preservatives, there was a better distribution on the dewar flask hays and less loss by evaporation, these probably being the main reasons why lower levels of organic acids preserved similar hays in dewar flasks than in bales. Also

to be considered is the fact that in dewar flasks the chemicals were diluted considerably with water before application to the hay whereas they were applied undiluted onto baled hay, and diluting organic acids with water can enhance their preservative activity (Herting et al 1974b).

Despite these differences it was felt that these dewar flask results were useful for comparing preservative treatments because they indicated the extent of inactivation of preservative on hay and although similarly treated hays exhibited different results, as shown by experiment one (table 22 fig 25), similar if not greater errors were apparent when using field trials to compare treatments for baled hay.

A similar sequence of events to that described above probably occurs when damp, organic acid treated baled hay deteriorates during storage and it would therefore appear that unless very high levels of organic acids are applied to the hay before baling, eventually the preservation would cease which would be unsatisfactory because baled hay is often stored for periods of up to six months.

A possible solution to this problem would be to treat the hay and then store it so that air could circulate between the bales and gradually dry the hay until it no longer required a preservative. However, there is the possibility that the volatile fatty acids would also evaporate in this situation and therefore the inclusion of a non-volatile preservative e.g. sorbic acid or propyl hydroxybenzoate with the volatile fatty acids could be useful in such a situation provided it was applied in sufficient quantities to preserve the hay until it had dried to approximately 20% moisture, after which it would not deteriorate.

C) The use of field trials

There were four main objectives of these field trials.

i) To determine the most accurate and convenient method of evaluating the extent to which the hay had deteriorated.

The advantages and disadvantages of using microbial counts and temperature recordings to estimate the extent of hay deterioration have previously been discussed, however, this topic will be considered further in connection with the field trial results, relating observed changes to the moisture content of the hay at baling.

The 15% moisture hay baled in the first field trial had not deteriorated or heated and appeared to be good hay after storage (table 31 fig 43).

The thermophilic fungal spore counts and the actinomycete spores, incubated at 37°C, counts decreased by significantly ($P<.05$) more in the treated hays than in the untreated, probably because the acid applied to the hay had caused a loss in the viability of some spores during storage, noting that results from the dewar flask experiments suggest that organic acids remain in hay if it does not deteriorate.

Considering the 19% moisture hay baled in the first field trial (table 31 fig 44) the untreated bales heated whereas the Hay Shield treated bales did not heat. This heating was considered to be mainly due to plant respiration as no changes in the microbial spore counts were detected.

The 19% moisture hay baled in the second field trial also heated without apparent microbial activity as assessed by spore counts and visual appearance (table 32, figs 45 and 46), although the thermophilic actinomycete spore numbers increased to approximately 10^5/g hay during storage, these changes being difficult to explain as the hay did not heat sufficiently to allow the growth of these thermophiles. Unlike the 19% moisture hay in the first field trial this heating was not prevented by organic acid treatment and neither was the heating of the 22% moisture hay in the fifth field trial which had been treated with either 2.0% propionic acid or 2.0% of a propionic acid: water mixture. Despite the high application rates of acid this latter hay heated considerably (fig 49) although there were no visible signs of moulding after storage. The heating pattern suggested that plant respiration was responsible because the hay heated rapidly reaching a maximum after 24 hours after which it cooled, whereas microbial activity appears to require a minimum of two days to attain its maximum as can be seen from the temperature - time graphs for the other field trials. Gregory et al (1963b) noted that damp baled hay heated to an initial peak after 1 day then cooled and reheated. They concluded that the initial temperature peak was caused by plant respiration.

These results suggest that in hay containing approximately 20% moisture there will be plant respiration producing heat but that only minimal microbial activity will occur as assessed by both spore counts and visual appearance of the hay before and after storage. Greenhill (1959) however, stated that hay required a moisture content of between 30% and 40% for plant respiration to occur. This apparent contradiction could be due to the heat in these field trials being

produced in the damp patches that have been found to occur in hay bales (fig 16), although no patchy moulding was observed.

In the fifth field trial the fact that the 2.0% propionic acid treated hay, heated more than the 2.0% propionic acid: water mixture treated hay was possibly because it may have been a little damper and although propionic acid does inhibit plant respiration, its distribution was very poor (table 39), and therefore the heating could have occurred in the untreated and low treatment areas.

Considering the 32% moisture hay baled in the fourth field trial (table 35 fig 48), firstly it should be noted that both the microbial counts after six weeks storage and the temperature recordings for the 1.0% propionic acid: sorbic acid mixture treated hay should be considered with caution because the two bales used for these measurements appear to have had a higher moisture level at baling than the remaining eight bales, as discussed in the results section.

Most of the microbial changes in this field trial appear to have occurred during the first six weeks storage as would be expected because this period contained the heating phase. The thermophilic actinomycete spore counts showed the largest increases rising from 10^3/g hay to 7 x 10^5/g hay in the untreated hay, whereas all the treatments (fig 48) significantly reduced ($P < .05$) this increase, the 0.5% propionic acid treatment doing so by less than the other treatments. The treatments did not affect increases in other spore numbers except for the mesophilic fungi where the 2.0% propionic acid: sorbic acid mixture treatment reduced the increases.

The microbial counts in this experiment show some correlation with temperature measurements. With the 2.0% propionic acid treated hay, which heated the most as measured by degree days, having the highest thermophilic fungal spore counts. The low actinomycete spore counts on this hay could possibly have been because of the high levels of acid present (tables 36, 37 and 38, which had prevented actinomycete growth relatively more than fungal growth, due to a p.H effect.

Visual assessment of the hay agreed with the spore counts, especially those of the thermophilic actinomycetes, the untreated hay appearing whiter than the treated hays of which the 0.5% propionic acid treated hay appeared the whitest. It is of interest to note that mouldy hay appears white and that mycelium of many actinomycete species which commonly occur in hay is whiteon agar media, whereas the majority of fungal species common in deteriorated hay have darker spores and mycelium. Consequently a visual assessment of deterioration in hay could reflect actinomycete rather than fungal growth.

The 2.0% treatment with the propionic acid: sorbic acid mixture appears to have preserved the hay because after three months storage there had been no indication of deterioration and its moisture level had decreased to 22% (table 35) suggesting it was unlikely to deteriorate. The hay also had a strong smell of propionic acid after the three months storage.

The 42% moisture hay baled in the first field trial (table 31 fig 42) heated considerably to between 54°C and 65°C, the 0.4% and 0.8% Hay Shield treatments slightly reducing this heating.

Despite this considerable microbial activity, the Hay Shield treatments had reduced the increases in thermophilic actinomycete spores. Similar results were obtained in some dewar flask experiments (e.g. Experiment 6 table 27). where organic acid treatments had not prevented heating but had reduced thermophilic actinomycete spore increases. This could be because the acids had reduced the p.H of the hay which had caused a sufficient delay in the growth of the actinomycetes to reduce their final spore numbers after heating.

The visual assessment of this hay was similar for the untreated and treated hays as would be expected from the temperature recordings, although the correlation between the appearance of the hay and the thermophilic actinomycete spore count that was apparent in the fourth field trial did not occur in this hay.

Finally, the 44% moisture hay baled in the third field trial (table 33 fig 47) gave some inconsistant results. As expected from its high baling moisture content both the untreated and treated hays heated considerably. The microbial counts after 40 days storage also suggest considerable microbial activity had occurred in all the bales, the actinomycete spore counts increasing to 10^7/g hay and the fungal spore counts to between 5 and 10 x 10^6/g hay. These counts were higher than for the 32% moisture hay in the fourth field trial and the 42% moisture hay in the first field trial suggesting greater microbial activity had occurred. This greater activity was probably because the hay had a higher moisture content at baling and also it was a greener, fresher hay when baled suggesting it contained a higher nutrient content (Watson and Nash 1960).

The microbial counts after three months storage, however, show a different pattern. The thermophilic actinomycete and fungal spore counts were significantly higher ($P<.05$) in the untreated hay and the hay treated with 0.25% of the Hay Shield: sorbic acid mixture (96:4 w/w), than in the remaining treated hays. This was because the thermophilic actinomycete spore numbers had decreased in the 40 days to 3 months storage period in the treated hays, whereas they had remained relatively constant in the untreated hay and the hay treated with 0.25% of the Hay Shield: sorbic acid mixture. The mesophilic spore numbers also decreased in the treated but notin the untreated hays, whereas the thermophilic fungal spore numbers had increased in the untreated hay but remained constant in the treated hays. The thermophilic actinomycete spore numbers did not decrease in the 4th field trial during the six week to three months storage period, where, even though the hay heated and the microbial spore counts increased, a high proportion of the applied acid remained after storage (tables 37 and 38 . Although final organic acid level determinations were not made on the hay in the third field trial, table 37 and 38 suggest some of the applied acid may still have remained on the hay after six weeks storage and this acid may have caused the changes in spore counts between six weeks and three months storage. Similar changes did not occur in the fourth field trial because the spore counts were lower after six weeks storage. This would explain why the actinomycete spore numbers were reduced whereas the fungal spore numbers were relatively constant in the treated hays, fungi preferring more acidic conditions than actinomycetes.

Generally, in these field trials the microbial spore counts correlated with both the visual appearance of the hay and the heat

production in the hay, although thermophilic actinomycete spore number increases were frequently reduced by treatments where no decrease in heating had been observed. Where hay has been treated with organic acids and has then deteriorated it seems possible that when heating has finished, sufficient acid may remain to cause a loss in viability of many of the spores present.

It would appear that in order to estimate the deterioration of hay, recording temperature changes and then visually examining the hay after storage would be sufficient. The estimation of microbial spore counts appears unnecessary as it yields little additional information and it is safe to assume that if hay has heated to 45°C or above it will contain many hazardous microbial spores.

ii) Determination of the practical problems concerned with the chemical preservation of hay

From the results obtained in these field trials the most important practical problems concerned with the organic acid preservation of hay using present day methods appear to be:-

1) Poor distribution of preservative.

2) Loss of preservative from the hay during storage.

3) No rapid and accurate means of estimating the moisture content or preferably the water activity of hay at baling, and a lack of information relating this to the minimum level of preservative necessary to prevent deterioration.

1) Poor distribution of preservative

This is probably the most important problem.

The poor distribution of organic acids on treated hay in bales is shown by the high values for the coefficients of variation for the results in tables 36 and 39'. Because these values are higher in table 39 (5th field trial immediately after baling) than in table 36 (4th field trial immediately after baling) it would appear that a multi-jet application system would give a slightly better distribution of preservative than a single jet system, although it is still not ideal.

This evidence for poor preservative distribution is supported by results obtained using the fluorescent dye suspensions, examples of which are shown in photographs 1 and 2, where patches of dye can be observed.

Kepner et al (1972) discussed factors affecting the spraying of liquids and noted that the viscosity, surface tension and application pressure of the liquid all affect the spray pattern. The primuline solution and to a greater degree the fire orange suspension, because it is not a true solution, used in this research will probably have different physical properties to the organic acids and therefore they would give different spray patterns using the same application equipment. Unfortunately these fluorescent dyes cannot be added to the organic acids because the acidity quenches their fluorescence as shown for primuline in table 13 and for dye suspensions including fire orange by Sharpe (1974). It was considered, however, that these differences were not of great importance in these studies because they would affect the properties of the spray droplets rather than the overall spray distribution, the latter being the important consideration for hay preservation.

2) Loss of preservative from hay during storage

The results from the dewar flask experiments and the 2nd field trial (table 32) suggest that organic acid levels rapidly decrease when plant or microbial activity raises the temperature of treated hay. In contrast the results for the fourth field trial before and after six weeks and three months storage (tables 36, 37 and 38) show that the organic acid levels were only slightly reduced during storage.

The reason for this apparent increased persistance of these acids on baled hay when compared with similar hay stored in dewar flasks, is unclear. One possible explanation is that because water cannot diffuse out and evaporate the moisture content of hay stored in dewar flasks tends to increase during storage, due to microbial metabolic water production, whereas water levels decrease in bales (Table 35) due to evaporation. Therefore, there will be greater bacterial activity in dewar flask hay. The results in fig 39 show that some bacterial species have organic acid resistance and are therefore probably mainly responsible for their degradation.

Despite the fact that the organic acid levels did not decrease considerably on the hay baled in the 4th field trial, microbial activity still occurred, suggesting either that the acids were present at levels too low for preservation or that they had chemically combined with components of the hay rendering them at least partly inactive.

The percentage recoveries of organic acids shown in tables 36,

37, 38 and 39 were calculated from the applied level of acid. The results in table 36 show considerable variation in these recoveries ranging from 12% for hay treated with 2.0% propionic acid, to 50% for hay treated with 1.0% of the propionic acid; sorbic acid mixture. This inconsistency suggests large sampling errors due to the uneven distribution of the acids.

The results for the 1.0% propionic acid treated hay in tables 37 and 38 suggest that the propionic acid level increased from 0.029% after 6 weeks storage to 0.61% after three months storage. This apparent increase in propionic acid was probably caused mainly by sampling errors, although it could have been partly because the bale examined after three months storage had had more acid applied and had been drier at baling and therefore had deteriorated less during storage, which in turn had led to less acid degradation.

3) Relationship between moisture content, water activity and preservative levels on hay.

The important role that water plays in the deterioration of hay has been well demonstrated in the literature (Gregory et al 1963b, Festenstein et al 1965 Currie and Festenstein 1971) and by results obtained in this research. Therefore any work on the microbial spoilage of hay and its chemical prevention will require accurate determinations of its moisture content.

Considering the addition of preservatives, it is clear that the higher the moisture content of the hay, the more preservative that will be necessary for control of spoilage, which has been shown to

apply for grain (Huitson 1968, Sogn 1973) and for hay stored in dewar flasks (fig 34). Therefore the moisture content of hay needs to be known before the minimum effective level of preservative can be applied.

The method for determining the moisture content of hay that first comes to mind is oven drying, however, fig 14 shows that care is needed in selecting the drying temperature as values above 80°C appear to cause the loss of other volatile components of the hay. The levels of these volatile compounds are higher in deteriorated hay than in fresh hay (Fig 14) suggesting they are due to microbial activity and are probably mainly volatile nitrogen compounds.

Assuming the hay is dried at 80°C, the main disadvantage of oven drying is that it requires at least 36 hours to give an accurate value (fig 15) which is obviously too long to be practical with hay that is to be baled, because in good haymaking conditions hay loses its water rapidly in the field (Burroughs et al 1967). Therefore to calculate the minimum effective application rate of preservative the moisture content of the hay has to be calculated rapidly.

During the field trials a selection of moisture meters were used, checking their results against those obtained by oven drying the same hay samples. All these meters were found to be inaccurate especially for damper hays (over 30% moisture), probably because they measured the electrical conductance of the hay to estimate its moisture content and this depended on the ionic concentration of the hay moisture, which would have varied considerably between different types of hay.

There was, however, a moisture meter produced by R.D.S. Ltd., which gave estimates of hay moisture levels which agreed, within acceptable limits, with those obtained by oven drying (fig 16), the average difference over the twelve samples being 2.7% water. The use of such a moisture meter would improve hay making with preservatives for the reasons described above, providing sufficient work had been carried out, relating the moisture content of hay at baling with the minimum effective level of preservative.

The problem then arises, that various types of hay at the same moisture content often require different levels of preservative for control, as has been shown in this work using hay stored in dewar flasks (fig 34). This variation probably occurs because hay that has been lying in the field loses some nutrients due to leaching by rain (Watson and Nash 1960), therefore fresh green hay would provide a richer nutrient medium for the activity of micro-organisms and plant enzymes.

In contrast, the more soluble carbohydrate that is present for a given amount of water, the lower the water activity of the hay.

As has been discussed in the introduction, when considering the deterioration of hay, it is the quantity of water available for microbial growth, expressed as water activity, which is important, and not the actual amount of water present.

Therefore when assessing the minimum effective quantity of preservative, the water activity of hay needs to be estimated in preference to the moisture content, and the percentage by weight of readily available nutrients also needs to be considered, the last

value probably being related to the length of time the hay has been lying in the field and the amount of rain it has received.

Ayerst (1965) reviews the role of water in deterioration and from his work it appears the most suitable method for determining the water activity of hay would be to equilibrate samples with air in a closed chamber at a constant temperature and then to measure the dew point or relative humidity of that air. What is not known is the time period it would take for the system to reach equilibrium, but this time could be shortened by chopping up the hay and continually mixing it with the air. Ayerst states that with reasonable care this method would measure water activity + 0.01 Aw which would be sufficient accuracy for this requirement.

The main problems in estimating the water activity of hay, appear to be, obtaining a short equilibration time and the use of small samples would probably be necessitated, therefore the recurring problem of sampling errors arises.

iii) A comparison of the results obtained from the dewar flask experiments with results from the field trials, in order to determine the minimum effective application rates of organic acids on baled hay at various moisture contents.

The factors which cause differences between the deterioration of hay in dewar flasks and in bales have been discussed above.

The fourth field trial is the only one where a chemical treatment preserved hay that would otherwise have deteriorated.

In this experiment 32% moisture hay was preserved by a 2.0%

treatment with the propionic acid: sorbic acid mixture. Although 2.0% propionic acid did not preserve this hay, it appears that 2.0% organic acid is approximately the minimum effective treatment level for 30% moisture baled hay, therefore, by comparison with fig 34 where approximately 0.4% organic acid was required for preservation of 30% moisture hay in dewar flasks, it can be concluded that baled hay requires an approximate five fold increase in the minimum organic acid level necessary for control, when compared with similar hay stored in dewar flasks.

If this calculation is applied to the results in fig 34 then the minimum organic acid levels necessary for the preservation of baled hay at different moisture levels, as shown in table 43, are obtained.

Moisture content of hay at baling	Minimum preservative level of propionic acid.
30%	2.0%
35%	3.5%
40%	5.0%
45%	7.0%

Table 43 - relationship between the moisture content of hay at baling and the minimum level of propionic acid necessary for preservation.

It should be noted, however, that these results are subject to considerable variations, for reasons outlined previously, and which can be seen from the results in fig 34. It is also possible that at higher preservative application rates on baled hay, factors including, improved distribution and greater diffusion through the hay would decrease the difference between hay stored in dewar flasks

and hay stored in bales.

iv) Possible improvements on the present day chemical preservation of hay

The problems concerned with the chemical preservation of hay have previously been outlined. Therefore, the question remains of how to solve these problems and hopefully create the situation where the deterioration of baled hay can be controlled using preservative levels similar to or only slightly higher than those necessary to preserve similar hay stored in dewar flasks.

Firstly, the present day methods of hay-making are considered and possible sources of error are outlined.

When grass is cut in the field it has a low microbial spore count (Table 31) and the microflora present at this time consists of species different to those which are dominant during storage. The fungal flora consists of Cladosporium spp, Fusarium spp and Alternaria spp and the few thermophilic or thermotolerant fungi present are almost entirely Aspergillus glaucus spp.

After cutting, the grass is left in the field being turned occasionally and if good weather persists, it will be ready for baling (moisture content less than 20%) in two to three days. There is little microbial activity in the grass during this period and the spore counts decrease (Gregory et al 1963b, table 31) probably due to ultra-violet radiation from the sun.

Difficulties arise if the drying conditions are poor before the

hay has dried sufficiently because if the hay is re-wetted then microbial activity starts and nutrients are leached out (Watson and Nash 1960). The microbial population will be growing vegetatively and as such will require higher levels of preservative for inhibition than spores (Thornton 1963), the latter probably being the main source of inoculum on fresh hay.

Eventually the farmer decides to bale the hay before too much nutrient is leached out, however, if the hay is above 20% moisture it will require preservation. The farmer therefore decides to apply a chemical preservative.

While it is in the field the hay is lying unevenly, therefore the baler picks it up unevenly but the preservative is being applied at a constant rate thus creating a problem of poor distribution. The situation is worsened by the fact that the clumps of hay which will receive the least preservative are probably the wettest and consequently damp patches occur in the bale (fig 36) containing little preservative. Deterioration will start in these regions creating patches of moulding as have been observed in deteriorating grain (Christensen and Gordon 1948 Lacey 1971a). The micro-organisms will spread from these areas, degrading preservative as they do so and producing heat which would volatolise some preservative allowing it to diffuse more rapidly out of the bale.

Present application systems consist of a series of jets mounted above the pick-up of the baler and it is obvious that only one side of the hay will be treated as it enters the baling chamber, this probably being the more important reason for the poor preservative distributions that have been obtained (Tables 36 and 39).

From this brief outline of the present day method and from the previous discussion, there appear to be four areas where improvements can be made in the chemical preservation of hay.

The first step would be to encourage good farming practice ensuring the hay dries as evenly as possible and lies as evenly as possible in the field so that it is picked up by the baler at a constant rate.

The second stage would be to improve the application equipment, the ideal solution being to design a machine whereby the hay could be tumbled in a mist of preservative immediately before baling, so that each piece of hay would receive a fine coating of preservative. Alternatively if the hay could be turned and mixed as it passed under a series of fine jets the preservative distribution would be improved.

Thirdly, the measurement of the water content and water activity of hay and their relationship with the quantity of preservative necessary for control have previously been discussed.

Fourthly, can the formulation of the preservative be improved? The evidence in this work suggests that propionic acid is as practical as any other compound or mixture of compounds. The inclusion of sorbic acid may improve it slightly but whether the extra expense involved would be justified is doubtful. By comparing results in fig 24 with those in tables 15 to 18 it seems possible that a mixture of a strong mineral acid and propionic acid (1:1 w/w) applied to hay at 2.0% would be a more effective preservative than 2.0% propionic acid alone, because the extra lowering of the p.H of the hay by the

mineral acid would enhance the anti-microbial activity of the 1.0% propionic acid sufficiently for it to be greater than that of 2.0% propionic acid alone at the higher p.H value. However, there is no evidence that this would occur in the bale and practical problems would arise, from the use of strong mineral acids, in the forms of machinery corrosion and danger to workers.

Knapp et al (1974) obtained encouraging results by injecting anhydrous ammonia into hay and noted that this compound could be used to reduce the heating of hay that was already at a high temperature. It is possible that the use of volatile preservatives could become more significant in the chemical preservation of hay because they would help to overcome the distribution problem.

The results from this investigation suggest there is a need to concentrate further research, in the use of chemical preservatives for hay, in three main areas.

Firstly, after baling a series of growth requirements in the hay determines the microbial activity that will occur. The micro-organisms start to grow and as they do so, they cause changes, both physical and biochemical, to occur within the hay, which in turn affect further microbial growth. These interactions continue until deterioration ceases. A better understanding of these complex changes and interactions is required and then possibly from this information, improvements could be made in the formulation of a chemical preservative.

Secondly, to prevent the occurrence of patches of deterioration

within a bale of treated hay, the development of preservative application equipment is needed to improve the distribution of preservative and farmers should be encouraged to dry the hay as evenly as possible.

Thirdly, improvements in the formulation of hay preservatives, although of some value, would be relatively unimportant until the application equipment had been improved, because an effective preservative would be unlikely to prevent microbial activity unless it was applied evenly to the hay. The use of gaseous or volatile preservatives e.g. ammonia and sulphur dioxide, could help to overcome the distribution problem, although such compounds are generally unpleasant to handle and there is limited information available on their efficiency as hay preservatives.

In Great Britain approximately 14.5 million tons of hay is baled annually. If, for example, on average, a 1% treatment with a preservative would be necessary to ensure none of this hay deteriorated, then 145,000 tons of that preservative would be required each year. Therefore, any chemical considered as a hay preservative would have to be available in large quantities. However, it can be seen that a commercially viable, and effective method for the chemical preservation of hay could possibly be a considerable prize for the agro-chemical industry.

REFERENCES

ALLEN M.B. (1953) - The thermophilic aerobic sporeforming bacteria. Bact. Rev. 17 125.

ALPINIS A.E. (1963) - Occurrence of thermophilous microfungi in certain alluvial soils near Nottingham - Nova Hedwigia 5 57 - 78.

ANDERSON A.A. (1958) - New sampler for the collection, sizing and enumeration of viable airborne particles. J. of Bact. 76 471

AUSTWICK P.K.C. (1963) - Ecology of Aspergillus fumigatus and the pathogenic phycomycetes. Recent Progress in Microbiology 8 644.

AYERST G. (1965) - Water activity, its measurement and significance in biology. Int. biodet. bull. 1 13 - 24.

AYERST G. (1969) - The effects of moisture and temperature on growth and spore germination of some fungi - Int. stored products Research 5 127 - 141.

BAGINSKI M. BAHRECKE G. LUCK E and D. GROLL (1973) - Preparation and method for the preservation of cereal grains. Brit.Patent 1316546 London.

BEDFORD P.G.C. and E.G.C. CLARKE (1973) - A preliminary study of the suitability of sorbic acid for use as a preservative in cat food preparations Vet. Rec. 92 55

BELL T.A. ETCHELLS J.L and A.F. BORG (1959) - Influence of sorbic acid on the growth of certain species of bacteria, yeasts and filamentous fungi - J. Bacteriol. 77 573 - 580.

BENHAM C.L. JAMIESON M, GREIG D.J. and C.D. RICKABY (1975) - propionic acid treatment of hay - Min of Agric. Fish. and Food. Agr. Developement and Advisory Service - North Region Rep. 1.667.

BENNET E.O (1973) - Formaldehyde preservatives for cutting fluids - Int. biodet. bull. 9 95- 100.

BERNIER C.C. (1973) - Chemical control of fungi and bacteria in stored high-moisture feed grains Ann. technol. Agricole 22 587 - 593.

B.P. Chemicals patent No. L155485 (1969a) - Inhibition of mould growth in crops and animal feedstuffs.

B.P. Chemicals patent No. L160430 (1969b) - Inhibition of mould growth on crops and animal feedstuffs.

B.P. Chemicals patent No. 11257123 (1971) - Inhibition of mould or bacterial growth on foodstuffs.

BRANCATO F.P and GOLDING N.S. (1953) - The diameter of the mold colony as a reliable measure of growth - Mycologia 45 848 - 864.

BROCK T.D. and FREEZE H. (1969) - Thermus aquaticus gen n. and sp.n - a nonsporulating extreme thermophile. J. Bacteriol 98 289 - 297.

BROWN D.C. and VALENTINE (1972) - Formaldehyde as a silage additive 1. The chemical composition and nutritive value of frozen lucerne, lucerne silage and formaldehyde - treated lucerne silage Aust. J. Agr. Res. 23 1093

BURMEISTER H.R., HARTMAN P.A. and R.A. SAUL (1966a) - Microbiology of ensiled high-moisture corn - Appl. Microbiol. 14 31 - 34.

BURMEISTER H.R. and P.R. HARTMAN (1966b) - Yeasts in ensiled high - moisture corn. Appl. Microbiol 14 35 - 38.

BURRELL N.J. KOZAKIEWICZ Z ARMITAGE D.M. and J.H. CLARKE (1973) - Some experiments on the treatment of damp maize with propionic acid - Ann. Technol. Agricole 22 595 - 603.

BURROUGHS F.J. HUGHES R and W.B. EVANS (1967) - Crop resistance to drying - Report welsh Plant Breeding station 1966.

CALLELY A.G. and D. LLOYD (1964) - The metabolism of propionate in the colourless alga - Prototheca Zopfii - Biochem. J. 92 338 - 345.

CANDLISH E, CLARK K.W. and J.R. INGALLS (1973) - Organic acid treatment of hay - Can. J. Anim Sci. 53 513 - 518.

CANE F.M. HANSEN M. YODER R. LEPLEY K and P. COX (1971) Effect of processing on salmonella moulds and other harmful substances in feeds. Feedstuffs 43 22 - 24.

CHANG Y and H.J. HUDSON (1967a) - The fungi of wheat straw compost - 1 - Ecological Studies - Trans. Br. Mycol. Soc. 50 649.

CHANG Y (1967b) - The fungi of wheat straw compost 11 - Biochemical and Physiological Studies - Trans. Br. Mycol. Soc. 50 667.

CHAPMAN E.S. (1974) - Effect of temperature on growth rate of seven thermophilic fungi - Mycologia 66 542 - 546.

CHEN T.C. and E.J. DAY (1974) - Gentian Violet as a possible fungal inhibitor in poultry feed; plate assays on its antifungal activity - Poultry Sci. 53 1791 - 1795.

CHRISTENSEN C.M. and D.R. GORDON (1948) - The mold flora of stored wheat and corn and its relationship to heating of moist grain - Cereal Chemistry 25 40 - 51.

CLARK J.H. FROBISH R.A. HARSHBARGER K.E. and R.G. DERRIG (1973) - Feeding value of dry corn, ensiled high-moisture corn and propionic acid treated high-moisture corn fed with hay or haylage for lactating dairy cows - J. Dairy Sci. 56 1531 - 1539.

COCHRANE V.W. (1958) - Physiology of fungi - John Wiley and Sons - New York.

COLIN C.T. and L. STOLOFF (1974) - Implications of mycotoxins for human health - J. Agric Food Chem. 22 1006 - 1015

COONEY C.L. and D.W. LEVINE (1972) - Microbial utilisation of methanol - Adv. in Appl. Microbiol. 15 337 - 365.

COONEY P.G. and R. EMERSON (1964) - Thermophilic Fungi - Eumyata, San Francisco - W.H. Freedman Publ. Co.

CORBAZ R. GREGORY P.H. and M.E. LACEY (1963) - Thermophilic and mesophilic Actinomycetes in mouldy hay - J. Gen. Microbiol 32 449.

CRAWFORD R.L. (1975) - Degradation of 3 - hydroxybenzoate by bacteria of the genus Bacillus - Appl. Microbiol. 30 439 - 444.

CRISAN E.V. (1973) - Current concepts of thermophilism and the thermophilic fungi - Mycologia 65 1171 - 1198.

CROOK P. CARPENTER C.C. and P.F. KLENS (1950) - The use of sodium propionate in isolating actinomycetes from soil - Science 112 656.

CROSS. T. (1968) - Thermophilic actinomycetes - J. Appl. Bact. 31 36.

CROSS T. and M. GOODFELLOW (1973) - Taxonomy and Classification of the Actinomycetes p. 11 in Actinomycetales Characteristics and Practical Importance Ed. Sykes G and Skinner F.A. Academic Press.

CROSS T. and A.M. MaCIVER (1968) - The Thermophilic Actinomycetes in mouldy hay - Micropolyspora faeni sp Nov. - J. Gen. Microbiol 50 351.

CURRIE J.A. and G.N. FESTENSTEIN (1971) - Factors defining spontaneous heating and ignition of hay. J. Sci. Fd. Agric 22 223 - 230.

CZERKAWSKI J.W. (1974) - Methods for determining 2 - 6 diaminopimelic acid and 2 - aminoethylphosphoric acid in gut contents. J. Sci. Fd. Agric 25 45 - 55.

DANZIGER M.T. STEINBERG M.P. and A.I. NELSON (1973) - Effect of cO_2, moisture content and sorbate on safe storage for wet corn - Trans. Am. Soc. Agric. Engin. 16 679 - 682;

DAVIS N.D, WAGENER R.E. MORGAN-JONES G and U.L. DIENER (1975) - Toxigenic thermophilic and thermotolerant fungi-Appl. Microbiol. 29 455 - 457;

DENNIS C (1975) - Effect of preharvest fungicides on spoilage of soft fruit after harvest - Ann. Appl. Biol. 81 227 - 234

DENNIS C. and A. GAUNT (1974) - Effects of Formaldehyde on fungi from boiler houses-J. Appl. Bact. 37 595 - 601.

DEPLOEY J.J. and C.L. FERGUS (1975) - Growth and Sporulation of thermophilic fungi and actinomycetes in O_2 - N_2 atmospheres-Mycologia 67 780 - 797.

DEUEL H.J. ALFIN -STALER R WEIL C.S. and SMYTH H.F. (1954a) - Sorbic acid as a fungistatic agent for Foods 1 - Harmlesseness of sorbic acid as a dietary component - Food Res. 19 1 - 12.

DEUEL H.J. CALBERT C.E. AMSFIELD L, McKEEHAN H. and H. BLUNDEN (1954b) - Sorbic acid as a fungistatic agent in foods 11. Metabolism of $\alpha.\beta$ - unsaturated fatty acids with emphasis on sorbic acid - Food Res. 19 13 - 17.

DEYOE C.W. RAO C.S. and R.P. KNAKE (1973) - Preservation of high - moisture grain using organic acids - Ann. Technol. Agricole 22 605 - 614.

DREW LO. KEENER H.M VAN KEUREN R.W, CONRAD H.R. and W.E. GILL (1974) - Preservatives for hay - Dep. Agric Eng. Ohio St. Univ. OH10 REPORT 59 38 - 39.

DRUCE R.G. and S.B. THOMAS (1970) - An ecological study of the Psychrotrophic bacteria of soil, water, grass and hay. J. Appl. Bact. 33 420.

DRUETT H.A. (1967) - The inhalation and retention of particles in the human respiratory system - 17th Symposium of Soc. for Gen. Microbiol 165 - 202.

DRYSDALE A.D. (1973) - The use of propionic acid for moist grain preservation in Britain - Ann. Technol. Agricole 22 615 - 619.

DUDMAN W.F. (1963) - Sorbic Hydroxamic acid - an antifungal agent effective over a wide p.H range - Appl. Microbiol 11 362 - 364.

DURBIN R.D. (1961) - Techniques for the observation and isolation of soil Micro-organisms. Bot. Rev. 27 522.

ECKERT J.W, and M.J. KOLBEZEN (1962) - Control of Penicillium decay of citrus fruits with 2 - amino butane Nature 194 888 - 889.

EGGINS H.O.W. and G.J.F. PUGH (1962) - Isolation of cellulose decomposing fungi from soil - Nature 193 94 - 95.

EGGINS H.O.W. and K.A. MALIK (1969) - The occurrence of thermophilic cellulolytic fungi in a pasture land soil - Ant. Van. Leewen 35 178.

EGGINS H.O.W. SZ1LVINYI A.V. and D. ALLSOPP (1972) - The isolation of actively growing thermophilic fungi from isolated soils. Int. Biodeterior. Bull. 8 53.

EKSTRÖM N. (1973) - Preservation of moist feed grain by treatment with organic acids - Ann. Technol. Agricole 22 621 - 629.

EL-AM A.S. (1975) - Variation in vivo and isolation of Aspergillus fumigatus from a case of human Aspergillosis - Mycologia 67 1114 - 1118.

ENEBO L. (1949) - Symbiosis in thermophilic cellulose fermentation Nature 163 805 - 811.

ENEBO L (1951) - On three bacteria connected with thermophilic cellulose formentation - Physiologia Plantarum 4 652 - 665.

EVANS H.C. (1972) - Thermophilous fungi isolated from the air-Trans. Br. Mycol. Soc. 59 516.

EYRE P (1972) - Pulmonary Emphysema - Vet. Rec. 91 134 - 140.

FAY J.P. and R.N. FARIAS (1975) - The inhibitory action of fatty acids on the growth of Eshcherichia coli - J. Gen. Microbiol 91 233 - 240.

FERGUS C.L. (1964) - Thermophilic and thermotolerant molds and actinomycetes in mushroom compost during peak heating - Mycologia 56 267.

FERGUS C.L. (1969) - Cellulolytic activity of thermophilic fungi and actinomycetes - Mycologia 61 120 - 129.

FESTENSTEIN G.N. (1966) - Biochemical changes during moulding of self-heated hay in dewar flasks. J. Sci. Fd. Agric 17 130.

FESTENSTEIN G.N. (1971) - Carbohydrates in hay on self-heating to ignition - J. Sci. Fd. Agric 22 231

FESTENSTEIN G.N. LACEY J and F.A. SKINNER (1965) - Self heating of hay and grain in Dewar flasks and the developement of Farmer's lung Antigens J. Gen. Microbiol 41 389.

FLOCKTON H.I. and T. CROSS (1975) - Variability in Thermoactinomyces vulgaris -J. Appl. Bact. 38 309 - 313.

FRANKENFELD J.W, KAREL M. and T.P. LABUZZA (1973) - Intermediate moisture food compositions containing aliphatic 1,3 - Diols -British Patent 1,409,024.

FRANKENFELD J.W. MOHAN R.R. and R.L. SQUIBB (1975) - Preservation of grain with aliphatic 1,3 - Diols and their esters -J. of Agr and Fd. Chem. 23 418 - 425.

FREESE E, SHEU C.W. and E. GALLIERS (1973) - Function of lipophilic acids as anti-microbial food additives - Nature 241 321 - 325.

GAMSON R.G. BOYD K.S. and J.W. LANE (1975) - Ultrastructural studies on Thermomyces lanuginosa and certain other closely related thermophilic fungi - Mycologia 67 961 - 971.

GIRISH G.K. GOYAL R.K. and K. KRISHANAMURTHY (1972) - Ethylene dibromide as a grain fumigant. J. Dairy Sci. 55 120 - 129

GOLUBCHUCK M. CUENDET L and W. GEDDES (1960) - Grain storage studies xxx - Cereal Chemistry 37 405

GOODING C.M. (1945) - Process of inhibiting growth of molds. U.S. Patent 2,379,294.

GOTTLIEB D and E.B. SHIRLING (1967) - Co-operative description of type cultures of streptomyces 1 - The International Streptomyces Project Int. J. System Bact. 17 315

GOUGH M.C. (1974) - The measurement of relative humidity with particular reference to remote long term measurement in grain silos - Tropical Stored Products Information 27 19 - 30.

GREENHILL W.L. (1959) - The respiration drift of harvested pasture plants during drying. J. Sci Fd. Agric. 10 495 - 501.

GREGORY P.H and M.E. LACEY (1962) - Isolation of thermophilic actinomycetes Nature 195 95

GREGORY P.H. and M.E. LACEY (1963a) - Mycological examination of dust from mouldy hay associated with Farmer's lung disease J. Gen. Microbiol 30 75 - 88

GREGORY P.H. LACEY ME, FESTENSTEIN G.N. and F.A. SKINNER (1963b) - Microbial and Biochemical changes during the moulding of hay - J. Gen. Microbiol 33 147

GREGORY P.H and M.E. LACEY (1963c) - Liberation of spores from mouldy hay - Trans. Br. Mycol Soc. 46 73

GREGORY P.H. FESTENSTEIN G.N. LACEY M.E. SKINNER F.A. PEPYS J. and P.A. JENKINS (1964) Farmer's lung disease. The developement of antigens in moulding hay - J. Gen. Microbiol 36 429

GRIGG D.B. (1974) - The agricultural systems of the world. Cam. Univ. Press.

HARRIS O. and M.L. FIELDS (1972) - A study of thermophilic aerobic sporeforming bacteria isolated from soil and water. Can. J. Microbiol 18 917

HEDGER J.N. and H.J. HUDSON (1974) - Nutritional studies of Thermomyces lanuginosa from wheat straw compost - Trans Br. Mycol Soc. 62 129 - 143

HENDERSON A.R. and P. McDONALD (1972a) - Effect of formic acid on the fermentation of grass of low dry matter content. J. Sci.Fd. Agric 22 157

HENDERSON A.R; McDONALD P. and M.K. WOOLFORD (1972b) - Chemical changes and losses during the ensilage of wilted grass treated with formic acid J. Sci. Fd. Agric. 23 1079

HENSSEN A. (1957) - Über die Beckutung der thermophilen mikroorganismen für die zersertzung des stallmistes. Arch. Mikrobiol 27 63 - 81.

HENSSEN A and S. EBERHARD (1967) - Studies on thermophilic actinomycetes Arch. Mikrobiol 57 214

HERTING D.C. and E.E. DRURY (1974a) - Antifungal activity of volatile fatty acids on grain - Cereal Chemistry 51 74 - 83

HERTING D.C. DRURY E.E. and S.R. AMES (1974b) - Antifungal activity of volatile fatty acids on graines 11. Effect of aqueous dilutions Cereal Chemistry 51 382 - 388

HOFFMAN C, SCHWEITZER T.R. and G. DALBY (1939) - Fungistatic properties of the fatty acids and possible biochemical significance - Food Res. 4 539 - 544

HUITSON J.J. (1968) - Cereal Preservation with propionic acid - Process. Biochem. 3 31 - 32

HUYSTEEN J.J. (1970) - The determination of short-chain fatty acids in aqueous solution by Gas liquid Chromatography. Water Res. 4 645 - 659

INGRAM M (1957) - Micro-organisms resisting high concentrations of sugars or salts - Microbial Ecology - 7th symposium Soc. Gen. Microbiol London. p.90 Camb. Univ. Press.

INGRAM M and E.M. Barnes (1956) - A simple modification of the deep shake tube for counting anaerobic bacteria - Lab. Practice 5 145

JAMES L.H. RETTGER L.F. and C. THORN (1928) - Microbial thermogenesis 11 - heat production in moist organic materials with special reference to the part played by micro-organisms. J. Bacteriol 15 117 - 141

JONES B.D. (1975) - Alflatoxin in feedingstuffs - its incidence, significance and control - Proceedings of the conference on animal feeds 1974 273-290.

JONES G.M. DONEFER E. and J.I. ELLIOT (1970) - Feeding value for dairy cattle and pigs of high-moisture corn preserved with propionic acid. Can.J.Anim Sci.50 483-489

JONES G.M. MOWATT D.N. ELLIOT J.I. and E.T. MORAN (1974) - Organic acid preservation of high - moisture corn and other grains and the nutritional value - a review. Can. J. Anim Sci 54 499 - 517

KAMINSKI E. STAVICKI S. WASOWICZ E and PRYZYBYLSKI (1973) Detection of deterioration of grain by gas chromatography - Ann. Technol. Agricole 22 401 - 407

KANE B.E. and J.T. MULLINS (1973) - Thermophilic fungi in municipal waste compost system - Mycologia 65 1087 - 1100

KARASZ A.B. and W. HALLENBECK (1972) - Preservatives and artificial sweeteners - simplified method for the determination of propionates in bread : Isolation by microdiffusion - J of Ass. Official. Analytical Chem. 55 4 - 6

KAUL J.L. (1972) - Effect of long storage after various treatments, on the viability and mycoflora of paddy seeds - Indian J. of Mycology and Plant Path. 2 156 - 159.

KEDDIE R.M. (1951) - Enumeration of Lactobacilli on grass and in silage - Proc. Soc. Appl. Bact. 14 157

KELLY D.P. (1970) - Metabolism of organic acids by Thiobacillus neapolitanus - Arch. Mikrobiol 73 177 - 192

KEPNER R.A. BAINER R. and E.L. BARGER (1972) - Principles of farm machinery A.V.I. Westport, Connecticut.

KLINNER W.E. and SHEPPERSON G. (1975) - The state of haymaking technology - A review. J. Br. Grassland Soc. 30 259 - 266

KNAPP W.R. HOLT D.A. and LECHTENBERG V.L. (1974) - Anhydrous ammonia and propionic acid as hay preservatives - Agronomy J. 66 823 - 824

KURDOGOCHI S. TAHARA S. and J. MIZUTANI (1974) - Fungal metabolites of sorbic acid - Agr and Biol Chem. 38 893 - 895

KUROGOCHI S. TAHARA S. and J. MIZUTANI (1975) - Fungal reduction of C_6 α-β— Unsaturated carboxylic acids - Agr. and Biol. Chem. 39 825 - 831

KUSTER E. (1972) - Simple working key for the classification and identification of named taxa included in the International Streptomyces Project. Int. J. of System. Bact. 22 139

KUSTER E. and R. LOCCI (1964) - Studies on peat and peat micro-organisms 11. Occurrence of thermophilic fungi in peat - Arch. Fur Mikrobiol 48 319 - 324.

KUSTER E. and S.T. WILLIAMS (1964) - Selection of media for isolation of Streptomyces - Nature 202 928

LACEY J. (1968) - The microflora of fodders associated with bovine respiratory diseases. J. gen. Microbiol 51 173

LACEY J (1971a) - The microbiology of moist barley storage in unsealed silos - Ann. appl. Biol. 69 187

LACEY J. (1971b) - Thermoactinomyces sacchari sp. nov a thermophilic actinomycete causing bagassosis. J. gen. Microbiol 66 327

LACEY J. (1973) - Actinomyces in soils, compost and fodders - The Soc. Appl. Bact. Symp. series 2 - ACTINOMYCETALES - Characteristic and practical importance Ed. G. Sykes and Skinner F.A. Academic Press - London

LACEY J. (1975) - Potential hazards to animals and man from micro-organisms in fodders and grain - Trans. Br. Myc. Soc. 65 171 - 184

LACEY J. and HILL M. (1969) - Rothamstead Report for 1969 p 139

LACEY J. PEPYS J and T. CROSS (1972) - Actinomycete and fungus spores in air as respiratory allergens - Safety in Microbiology No 6 p 151 Academic Press.

LECHEVALIER H.A. and M.P. LECHEVALIER (1967) - Biology of actinomycetes - Ann. Rev. Microbiol. 21 71

LEE B.H. and T.H. BLACKBURN (1975) - Cellulose production by a thermophilic Clostridium species - Appl. Microbiol 30 346 - 353

LEVVY G. and A. McALLAN (1959) - The N - acetylation and estimation of Hoxosamines - Biochem. J. 73 127.

LUCAS G.B. POUNDS J.R, SNOW J.P. (1973) - Mold control of stored tobacco with propionic and acetic acids - Tobacco Sci. 165 - 166

MAJUNDER S.K. RAG UNATHAN A.N. and J.R. RANGASWAMY (1973) - Control of microflora on moist grain - Ann. Techol. Agricole. 22 483 - 492

MARION P.T. RIGGS J.K. ARNOLD J.K. and J.L. ONEGER (1973) - Moist grain rations with volatile fatty acids for beef cattle - J. Animal Sci. 34 Abstract 100 p. 909

MARSH R.W. (1972) - Systemic fungicides - Longman - London.

MARUYAMA K and H. KITAMURA (1975) - Some effects of propionate on the growth of Rhodopseudomonas spheroides - Agr. Biol. Chem. 39 1521 - 1526

MATHEWS G.A. (1973) - Nozzles for pesticide application in the tropics P.A.N.S. 19 583 - 600

MAY K.R. (1945) - The cascade impactor - an instrument for sampling coarse aerosols - J. Sci. Instruments 22 187

McMAHON M.E, HARTMAN P.A. SAUL R.A. and L.H. TIFFANY (1975) - Deterioration of high-moisture corn. Appl. Microbiol. 30 103 - 109

McROBBIE D.I. and M.S. PARKER (1974) - Some aspects of the anti-fungal activity of esters of p-hydroxybenzoic acid-Int. Biodet. Bull. 10 109 - 112

MELNICK D. LUCKMANN F.H. and GOODING L.M. (1954) Sorbic acid as a fungistatic agent for foods VI. Metabolic degradation of sorbic acid in cheese by molds and the mechanism of mold inhibition - Food Res. 19 44 - 58

MILWARD Z (1976) - Further experiments to determine the toxicity of propionic acid to fungi infecting stored grain - Trans. Br. Mycol Soc. 66 319 - 324

MIROCHA C.J. HARRISON J. NICHOLLS A.A. and M. McCLINTOCK (1971) - Detection of a fungal oestrogen (F - 2) in hay, associated with infertility of dairy cattle. Appl. Microbiol. 16 797

MISLIVEC P.B. DIETER C.T. and V.R. BRUCE (1975) - Effect of temperature and relative humidity on spore germination of mycotoxic species of Aspergillus and Penicillium - Mycologia 67 1187 - 1189

MORGAN W. and L. ELSON (1934) - A colourimetric method for the determination of N - acetyl glucosamine and N - acetyl chondrosamine - Biochem. J. 28 988

MUKHERJEE S. (1952) - studies on degradation of fats by micro-organisms 111 - the metabolism of butyric acid by Aspergillus niger - Arch. Biochem. 35 34 - 59

MULINGE S.K. and C.G.C. CHESTERS (1970a) - Ecology of fungi associated with moist stored barley grain - Ann. Appl. Biol. 65 277 - 284

MULINGE S.K. and C.G.C. CHESTERS (1970b) - Methods of isolating the microflora of moulding high - moisture barley in partially sealed silos. Ann. Appl. Biol. 65 285

NIEMAN C. (1954) - Influence of trace amounts of fatty acids on the growth of micro-organisms - Bact. Rev. 18 147 - 163

OFOSU & ASIEDU and R.S. SMITH (1973) - Some factors affecting wood degradation by thermophilic and thermotolerant fungi - Mycologia 65 87

OKAZAKI H. and H. IIZUKA (1970) - The lysis of living mycelia of a thermophilic fungus Humicola lanuginosa by the cell wall-lytic enzymes produced from a thermophilic actinomycete-J. Gen. Appl. Microbiol 16 537

PEPYS J. JENKINS P.A. FESTENSTEIN G.N. LACEY M.E, GREGORY P.H, F.A. SKINNER (1963) Farmer's lung, thermophilic actinomycetes as a source of Farmer's lung hay antigen-Lancet 11 607 - 611

PHILIPS D.E. (1974) - The estimation of fungal growth in solid substrates - PhD thesis Univ Bath. England

PIZARRO E.A, DAVIES M.H, AND I.B. WARBOYS (1973) - Effect of different levels of propionic acid on the growth of Micropolyspora faeni (A.94) and Thermoactinomyces vulgaris (A.64) J. Sci.Fd. Agric 24 1351

PRICE J.S. and R. STORCK (1975) - Production, purification, and characterisation of an extracellular chitosanase from streptomyces J. Bact. 124 1574 - 1585

RAO C.S. KNAKE R.P. DEYOE C.W. and G.L. ALLEE (1974) Preserving high-moisture grain with organic acids - Feedstuffs 46 41 - 43

REISS J. (1970) - A simple cultivation chamber for the study of aerial reproductive elements of fungi - J. Gen. Appl. Microbiol. 16 185

RIDE J.P. and R.B. DRYSDALE (1972) - A rapid method for the chemical estimation of filamentous fungi in plant tissue - Physiol. Pl. Path. 2 7 -15

ROSENBERG S.L. (1975) - Temperature and p.H optima for 21 species of thermophilic and thermotolerant fungi - Can. J. Microbiol. 21 1535 - 1540.

ROTHBAUM H.P. (1961) - Heat output of thermophiles occurring on wool J. Bact. 81 165

ROTHBAUM H.P. (1963) - Spontaneous combustion of hay - J. appl. Chem. 13 291

RUBBO S.D. GARDNER J.F. and R.L. WEBB (1967) - Biocidal activities of glutaraldehyde and related compounds - J. appl. Bact. 30 78 - 87

SAKAGUCHI K, KURANE R.M. MURATA (1975) - Assimilation of formaldehyde and other C, compounds by Gliocladium deliquescens and Paecilomyces varioti - Agric and Biol. Chem. 39 1695 - 1702

SAUER F. (1973) - Control of mold by chemical preservatives - Agricultural Experimental Station - Geneva special report, New York State 13 22 - 24

SCOTT W.J. (1957) - Water relations of food storage micro-organisms - Adv. Food. Res. 7 83 - 96

SHARP R.B. (1974) - Spray deposit measurement by fluorescence - Pest. Sci. 5 197 - 209

SHEU C.W. and E. FREESE (1972) - Effects of fatty acids on growth and envelope proteins of Bacillus subtilis J.of Bact. 111 516 - 524

SHIRLING E.B. and D. GOTTLIEB (1966) - Methods for characterisation of streptomyces species - Int. J. System.Bact. 16 313

SHIRLING E.B. and D. GOTTLIEB (1968a) - Cooperative description of type cultures of streptomyces II species descriptions from first study Int. J. System. Bact. 18 69

SHIRLING E.B. and D. GOTTLIEB (1968b) - Cooperative description of type cultures of streptomyces III - additional species descriptions from first and second studies. Int. J. System.Bact. 18 279

SHIRLING E.B. and D. GOTTLIEB (1969) - Cooperative description of type cultures of streptomyces IV species descriptions from the second, third and fourth studies. Int. J. System. Bact. 19 391

SHIRLING E.B. and D. GOTTLIEB (1972) - Co-operative description of type strains of streptomyces V additional descriptions. Int. J. System. Bact. 22 265

SKOULAS A. WILLIAMS N. and J.E. MERRIMAN (1964) - Exposure to grain dust II a clinical study of the effects J. of Occup. Med. 6 359

SMITH W.L. (1972) - Chemical treatments to reduce post harvest spoilage of fruit and vegetables - Bot. Rev. 38 411 - 445

SMITH D.P. and N. ROLLIN (1954) - Sorbic acid as a fungistatic agent for foods VII effectiveness of sorbic acid in protecting cheese - Food Res. 19 59 - 65

SMITH R.B. GRIFFIN J.M. and P.B. HAMILTON (1976) - Survey of aflatoxicosis in farm animals. Appl and Environmental Microbiol. 31 385 - 388

SMITH R.E. and K.R. STEVENSON (1975) - factors influencing the growth of fungi in high - moisture corn treated with propionic acid. Int. Biodet. Bull. 11 97 - 100

SOGN L. (1973) - trials with propionic acid as a preservative for fodder barley. Ann. Technol. Agricole 22 675 - 683

STOJANOVIĆ S. ZAKULA SOFIJA and R. CURCIC (1972) The effect of various preservative methods on nutritive value of corn grain - Veterinaria 21 505 - 510

STUTZENBERGER F.J. (1971) - Cellulase production by Thermonospora curvata isolated from municipal solid waste compost - Appl. Microbiol. 22 147 - 152

STUTZENBERGER F.J. (1972) - Cellulolytic activity of Thermomonospora curvata - nutritional requirements for cellulase production - appl. Microbiol 24 77

SULOCHANA S. ABDULLA P.K. and K.T. PUNNOSE (1970) - Association of mycotic agents with bovine abortions in Kerala - Kerala J. Vet. Sci. 1 1

SYPESTEYN A.K. (1972) - Effect on fungal pathogens - p. 132 - 155 System Fungicides Ed. R.W. Marsh Longman. London.

TANSEY M.R. (1971) - Agar - diffusion assay of cellulolytic ability of thermophilic fungi-Arch. Mikrobiol 77 1

TANSEY M.R. (1973) - Isolation of thermophilic fungi from alligator nesting material - Mycologia 65 594

TEH J.S. (1974) - Toxicity of short - chain fatty acids and alcohols towards Cladosporium resinae Appl. Microbiol. 28 840 - 844

TENDLER M.D. and P.R. BURKHOLDER (1961) - Studies on the thermophilic actinomycetes 1 - Methods of cultivation - Appl. Microbiol. 9 394

THOMAS S. and A.D. RUSSELL (1974a) - Studies on the mechanism of the sporicidal action of glutaraldehyde - J. appl. Bact. 37 83 - 92

THOMAS S and A.D. RUSSELL (1974b) - Temperature induced changes in the sporicidal activity and chemical properties of glutaraldehyde-Appl. Microbiol. 28 331 - 335

THOMKE S. and A. TIDEN (1973) - Moist barley treated with propionic, acetic, or formic acid in rations to growing pigs. Swedish J. of Agr. Res. 3 145 - 151

THORNLEY J. (1960) - The differentiation of Pseudomonas from other Gram -ve bacteria on the basis of arginine metabolism. J. appl. Bact. 23 37 - 41

THORNTON R.H. (1963) - Anti-fungal activity of fatty acids to Pithomyces chartarum (Berk and Curt) M.B. Ellis and other fungi - New Zealand J. of Agr. Res. 6 469 - 483

TOBAK H.H. and W.B. Cooke (1968) - Growth and metabolism of fungi in an atmosphere of nitrogen - Mycologia 60 115 - 140

TRENK H.L. and P.A. HARTMAN (1970) - Effects of moisture content and temperature on aflatoxin production in corn. Appl. Microbiol. 19 781

TRENK H.L. BUTZ M.E. and F.S. CHU (1971) - Production of ochratoxins in different cereal products by Aspergillus ochraceus Appl. Microbiol 21 1032

TROLLER J.A. and R.A. OLSEN (1967) - Derivatives of sorbic acid as food preservatives J. Fd. Sci. 32 228 - 231

TSAO P.H. (1970) - Selective media for isolation of pathogenic fungi - Ann. Rev. Phytopath. 8 157

TSUJI A. KINOSHITA T. and M. HOSHINO (1969) - Analytical chemical studies on amino sugars 11. determination of hexosamines using 3 - methyl -2-benzothiazolone hydrazone hydrochloride - Chem. and Pharm. Bull. 17 1505 - 1510.

VALENTINE S.C. and D.C. BROWN (1973) - Formaldehyde as a silage additive II The chemical composition and nutritive value of lucerne hay, lucerne silage, and formaldehyde and formic acid treated lucerne silages. Australian J. Agric. Res. 24 939

VELOSO J.R. HAMILTON P.B. and C.R. PARKHURST (1974) - The use of formaldehyde flakes as an antimicrobial agent in built up poultry litter. Poultry Sci. 53 78 - 83.

WALDO D.R. KEYS J.R. and C.H. GORDON (1975) - Paraformaldehyde compared with formic acid as a direct-cut silage preservative. J. Dairy Sci. 58 992 - 930

WALDO J.R. SMITH L.W. MILLER R.W. and L.A. MOORE (1969) Growth, intake and digestibility from formic acid silage versus hay - J. of Dairy Sci. 52 1609 - 1616

WALLHÄUBER K.H. (1974) - Antimicrobial preservatives in biologics - Drugs made in Germany 17 103 - 117

WALSH J.H. and C.S. STEWART (1969) - A simple method for the assay of the cellulolytic activity of fungi - Int. Biodet. Bull. 5 15

WALSH J.H. and C.S. STEWART (1971) - Effect of temperature, oxygen and carbon dioxide on cellulolytic activity of some fungi - Trans. Br. Myc. Soc. 57 75

WATSON R.D. (1976) - Trouble in store. New Scientist 70 No 997 170 - 172

WATSON S.J. and M.J. Nash (1960) - The conservation of grass and forage crops - Oliver and Boyd Ltd. Edinburgh

WELLS J.M. (1973) - Fungicide dips for controlling decay of carrots in storage for processing-Plant Dis. Rep. 57 697 - 700

WESTENDORP T. (1963) - Turning infrequently or often in hay making - Land bauwroorichting 11 642 - 645

WILAMOWSKI G. (1971) - Preservatives and artificial sweetners - collaborative study of the colourimetric determination of sorbic acid in cheese - J. of Ass. of Off. Anal. Chem. 54 663 - 665

WILSON D.M. HUANG L.H. and E. JAY (1975) - Survival of Aspergillus flavus and Fusarium moniliforme in high-moisture corn stored under modified atmospheres - Appl. Microbiol. 30 592 - 595

WOOD J.G.M. and J. PARKER (1971) - Respiration during the drying of hay. J. of Agr. Engineering Res. 16 179 - 191

WOOD N.A. (1968) - The use of formic acid as a prophylactic for moniliasis. Game Res. Ass. 8th Ann. Rep. 7 58

WOOD N.A. (1969) - Formic acid as a prophylactic for candidasis in Partridges. Vet. Rec. 85 78

WOODCOCK D. (1972) - Structure - activity relationships. p.34 - 85 in systemic Fungicides Ed. R.W. Marsh. Longman. London.

WOOLFORD M.K. (1972) - Some aspects of the microbiology and biochemistry of silage making. Herbage Abstracts 42 105 - 111

WOOLFORD M.K. (1975a) - Microbiological screening of the straight chain fatty acids (C_1 - C_{12}) as potential silage additives. J. of Sci. Fd. Agric. 26 219 - 228

WOOLFORD M.K. (1975b) - Microbiological screening of food preservatives, cold sterilants and specific anti-microbial agents as potential silage additives - J. of Sci.. Fd. Agric. 26 229 - 237

WORK E. and D.L. DEWEY (1953) - The distribution of α-β-diaminopimelic acid among various micro-organisms J. gen. Microbiol. 9 394 - 409

YU YU and THOMAS J.W. (1975) - Effect of propionic acid and ammonium isobutyrate on preservation and nutritive values of alfalfa haylage. J. Anim. Sci. 41 1458 - 1467.

ZIMMER E. (1973) - New methods in fodder conservation. Vaextodling (Plant Husbandry) 28 90 - 97.

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